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THE INHIBITION OF SULPHANILAMIDE ACETYLATION BY AROMATIC AND HETEROCYCLIC CARBOXAMIDES AND CARBOXHYDRAZIDES[1]

BY WILLARD J. JOHNSON

Abstract

The acetylation of sulphanilamide by pigeon liver extracts was found to be markedly inhibited by amides of salicylic, 5-bromosalicylic, 5-methylsalicylic, and 6-aminonicotinic acids, and by hydrazides of benzoic, salicylic, 4-amino-salicylic, nicotinic, and isonicotinic acids, as well as by *p*-aminosalicylic acid. The inhibition by salicylamide and isonicotinylhydrazide (isoniazid) was shown to be competitive in nature.

Introduction

The principal mode of inactivation of sulphanilamide (*p*-aminobenzene-sulphonamide) and its therapeutically-active derivatives in the animal body is by acetylation at the aryl amino group. The rate and extent of acetylation varies with respect to animal species and to different sulphonamide compounds (4, 5, 8, 9, 10). Acetylsulphonamides are inactive therapeutically, are usually more toxic than the parent compound, and are rapidly eliminated by the kidneys. Kidney damage due to crystallization of acetylsulphonamide in the kidney tubules has been frequently reported (6). From the point of view of sulphonamide therapy, the inactivation and loss of free sulphonamide by acetylation and subsequent excretion necessitates the administration of frequent doses in order to maintain therapeutic levels of the drug in the body.

It has been found that certain aromatic and heterocyclic carboxamides and carboxhydrazides decrease the destruction of sulphanilamide and its derivatives by inhibiting competitively their acetylation. The inhibitory action of these compounds on the acetylation of sulphanilamide by extracts of pigeon liver will be described here. Their effects on sulphonamide acetylation *in vivo* as manifested by increased blood levels of free sulphonamide and decreased levels of acetylsulphonamide will be the subject of a separate paper.

[1] *Manuscript received October 18, 1954.*
Contribution from Research Laboratories, Frank W. Horner Limited, Montreal, Que.

Materials and Methods

Acetylating System

Acetone-dried pigeon liver was prepared as previously described (3). The dry powder was extracted with a solution composed of 0.09 *M* potassium fluoride and 0.06 *M* potassium chloride (60 mgm. of powder per ml. of extracting fluid). Extraction was made by gradual addition of the solution to the powder in a porcelain mortar with constant grinding for five minutes. The resulting suspension was centrifuged at 3000 r.p.m. for 25 min., and the supernatant decanted through glass wool. One milliliter of pigeon liver extract was added to each reaction tube after all other additions had been made. In addition to pigeon liver extract, all tubes contained the following substances, in final concentration, to a total volume of 3 ml.: potassium phosphate buffer, pH 7.4, 0.02 *M*; potassium acetate 0.02 *M*; potassium citrate 0.02 *M*; sodium adenosine triphosphate 0.004 *M*; sulphanilamide, 412 μgm. (8×10^{-4} *M*); acetylation inhibitors as indicated in the tables. The tubes were incubated for 30 min. at 37° C. in the presence of air, without shaking. The reaction was stopped by the addition of 0.2 ml. of 3% tricholoracetic acid to each tube.

No increase in the rate of acetylation was obtained by the addition of Coenzyme A (CoA) preparations, thus apparently indicating that the crude pigeon liver extract contains an optimum concentration of CoA. Likewise, the addition of cysteine was found not to influence the rate of reaction. Citrate, however, invariably increased the reaction rate, probably by binding divalent cations which are known to be inhibitory. This system regularly yielded a 40% or greater acetylation of sulphanilamide in a 30-min. incubation period, when no inhibitor was added.

Sulphanilamide Determination

Sulphanilamide was determined by the method of Bratton and Marshall (1). The difference between the sulphanilamide content of the incubated tubes and unincubated control was taken as the extent of acetylation. Complete recovery of sulphanilamide added to the control tubes was obtained in the presence of all inhibitors used, thus indicating that the latter did not interfere with the determination of sulphanilamide.

Results and Discussion

During the preliminary screening process compounds structurally related to sulphanilamide were tested arbitrarily at 10^{-3} *M* concentration for inhibitory action on sulphanilamide acetylation. Compounds which inhibited significantly at 10^{-3} *M* were tested further to establish the concentration required to produce approximately 50% inhibition. Typical results are shown in Table I.

TABLE I

INHIBITION OF SULPHANILAMIDE ACETYLATION

Inhibitor	Inhibitor conc., M./l. × 10^{-4}	Sulphanilamide acetylated, μgm./30 min.	Inhibition, %
None	0	178	—
A. Carboxamides			
2-Hydroxy-5-bromobenzamide (5-bromosalicylamide)	1	69	61
o-Hydroxybenzamide(salicylamide)	10	79	56
2-Hydroxy-4-methylbenzamide (*o*-cresotamide)	10	74	58
o-Chlorobenzamide	10	178	0
p-Hydroxybenzamide	10	159	10
o-Ethoxybenzamide	10	160	10
6-Aminonicotinamide	10	89	50
B. Carboxhydrazides			
Benzoylhydrazide	20	62	65
o-Hydroxybenzoylhydrazide (salicylylhydrazide)	5	76	57
p-Hydroxybenzoylhydrazide	10	84	53
2-Hydroxy-4-aminobenzoylhydrazide (PAS hydrazide)	3	84	53
Isonicotinylhydrazide(isoniazid)	10	79	56
Nicotinylhydrazide	30	94	47
C. *p*-Aminosalicylate (PAS)	20	50	72

Experimental conditions are described under "Materials and Methods".

Effect of Carboxamides

o-Hydroxybenzamide (salicylamide) at 10^{-3} *M* inhibited acetylation by 56%. The fact that salicylic acid at the same concentration was completely inactive indicated that the amide group was involved in the inhibitory effect of salicylamide. Replacement of the *o*-hydroxy function of salicylamide by a chlorine atom resulted in loss of activity, and a marked decrease in activity was observed when the *o*-hydroxy group was shifted to the para-position, thus illustrating the contribution to inhibitory potency made by the *o*-hydroxy group of salicylamide. Masking the *o*-hydroxy function by alkyl substitution, as exemplified by *o*-ethoxybenzamide in Table I, also led to inactivation. Bromination in the 5-position of salicylamide resulted in a 10-fold increase in activity. As with salicylamide, the *o*-hydroxy group of 5-bromosalicylamide was crucial to its activity. *o*-Cresotamide (4-methylsalicylamide) inhibited acetylation to the same extent as did salicylamide.

6-Aminonicotinamide (a representative pyridinecarboxamide) showed activity equivalent to salicylamide, while nicotinamide and nicotinic acid at 10^{-3} *M* were inactive. It should be mentioned that 6-aminonicotinamide proved to be extremely toxic to rabbits and rats, at low dosage levels causing paralysis of the extremities and blindness. These effects are being investigated further.

Effects of Carboxhydrazides

Aromatic hydrazides appeared to have greater inhibitory potency than the corresponding amides. Thus, benzoylhydrazide at 2×10^{-3} *M* inhibited acetylation by 65%, while benzamide was inactive; the hydrazide of salicylic acid was twice as effective as salicylamide. The activating influence of the *o*-hydroxy group of salicylylhydrazide appeared less pronounced than in the case of salicylamide, as indicated by the fact that salicylylhydrazide was only twice as effective as its *p*-hydroxy isomer. The activity of salicylylhydrazide was further increased by amination in the 4-position.

Isoniazid (isonicotinylhydrazide), in which the carboxhydrazide group is *para* to the nuclear nitrogen, was more than three times as active as nicotinylhydrazide, which has the carboxhydrazide group in the *meta*-position. This difference between the activity of isoniazide and nicotinylhydrazide shows up in fields which are apparently quite unrelated. Thus, isoniazid is a powerful tuberculostatic agent, a property not shared by nicotinylhydrazide. Similarly, isoniazid at 7.5×10^{-5} *M* inhibited by 50%, non-competitively, the decarboxylation of histidine by cell-free extracts of *Clostridium welchii*, whereas nicotinylhydrazide was inactive at 10^{-3} *M* (unpublished results).

Nature of Inhibition

The nature of the inhibition by salicylamide and isoniazid, as representative of their respective groups, was investigated by the method of Lineweaver and Burk (7). The results, shown in Figs. 1 and 2, indicated that the inhibition

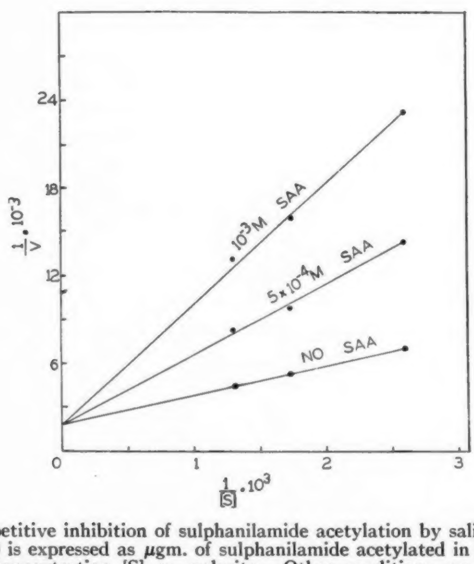

FIG. 1. Competitive inhibition of sulphanilamide acetylation by salicylamide (SAA). The velocity (V) is expressed as μgm. of sulphanilamide acetylated in 30 min., and the sulphanilamide concentration [S] as molarity. Other conditions are described under "Materials and Methods".

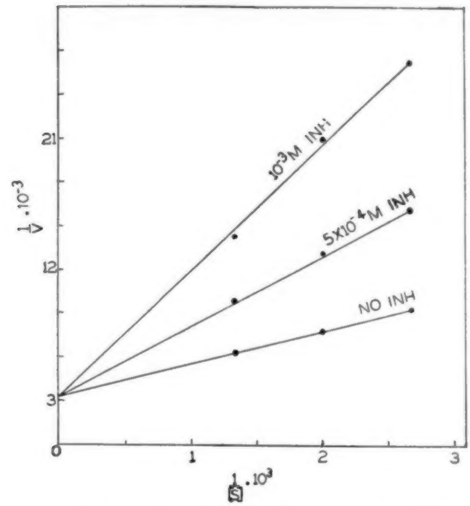

FIG. 2. Competitive inhibition of sulphanilamide acetylation by isoniazid (INH). Experimental conditions as for Fig. 1.

in both cases is obviously competitive. Moreover, isoniazid was found to be readily acetylated by pigeon liver extract (2) and thus is able to compete with sulphanilamide for available acetyl-CoA. It is probable that other hydrazides which inhibit sulphanilamide acetylation are similarly acetylated. There is no evidence as yet for acetylation of the carboxamide group, and in this case the inhibition may be due to the blockage of active centers of the acetylating enzyme.

Acknowledgments

I wish to express my thanks to Delmar Chemicals, Lachine, for generous supplies of many of the compounds used in this investigation. The technical assistance of Mr. Leslie Humber, and latterly, Mr. Julius Gordon, is gratefully acknowledged.

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AN INVESTIGATION OF THE COAGULATION DEFECT BROUGHT ABOUT IN RABBITS AND DOGS BY THE INTRAVENOUS INJECTION OF PAPAIN[1]

By Frank C. Monkhouse

Abstract

The intravenous injection of papain causes the blood of dogs and rabbits to become incoagulable. In rabbits the incoagulability persists for many hours. In some animals there remained a mild disturbance in the coagulation system up to two weeks after the injection. The release of heparin can account for part of the effect, but there is evidence of some other contributing factor. Results indicate that the added factor affects the thrombin–fibrinogen reaction. Calcium chloride in excess of that needed for normal coagulation will greatly inhibit the antithrombic effect of commercial beef heparin, and to a lesser extent, the antithrombic effect of an injection of papain.

Introduction

Eagle (3) and Ferguson and Ralph (4) investigated the action of papain on the coagulation system *in vitro*. They found that its action was similar to that of thrombin in that it could coagulate fibrinogen directly. Dyckeroff and Gigante (2) confirmed this and suggested the name "phyto-thrombin" to signify its plant origin.

In contrast to this *in vitro* coagulant action, Kellner *et al.* (6) showed that when papain was injected intravenously into rabbits the blood of these animals became completely incoagulable. They could not demonstrate abnormal amounts of antithrombin or anticoagulant. According to these authors the defect resembled hemophilia, and appeared to be due to a deficiency or inactivation of thromboplastin or a thromboplastin precursor.

In preliminary experiments it was found that papain, injected intravenously in doses of 20 to 30 mgm. per kilogram body weight, caused the blood of rabbits to become completely incoagulable. This incoagulability persisted for many hours after the injection. Contrary to the report of Kellner *et al.* (6), we found that, in every instance, there was an increase in antithrombin activity. A material could be extracted from the plasma and identified as heparin. There was some doubt in our minds, however, that the pronounced anticoagulant effect was entirely due to the presence of heparin. This was especially true in rabbits where the coagulation time was often increased out of proportion to the level of extractable heparin found in the blood. Even in dogs the heparin level had often returned toward normal, while the clotting time remained high.

These apparent discrepancies, and the suggestion by Kellner that the condition resembled hemophilia, encouraged us to undertake a further study of this interesting phenomenon. The following is a report of our study and the conclusions derived therefrom.

[1] *Manuscript received October 21, 1954.*
Contribution from the Department of Physiology, University of Toronto, Toronto, Canada. This work was supported by funds from the Defence Research Board of Canada, Grant No. 334.

Methods and Materials

Collection of Blood Samples

Unless otherwise stated, all samples were taken into one-ninth their volume of 3.8% (w/v) sodium citrate.

Heparin Estimation

Heparin was extracted by the method of Monkhouse and Jaques (9). The plasma was deproteinized with phenol and the heparin precipitated from the aqueous layer by the addition of two to three volumes of 95% alcohol. The mixture was allowed to remain at 2° C. for several hours to ensure complete flocculation of the precipitate. The material was then centrifuged and the precipitate washed, first with 95% alcohol, then ether, and finally dried at room temperature. The heparin was extracted from the dried powder by means of physiological saline. The extracts were then assayed according to their ability to prevent the coagulation of citrated dog blood or horse plasma on the addition of dilute solutions of thrombin. The potency is expressed in terms of standard beef heparin kindly supplied by the Connaught Medical Research Laboratories.

Aluminum Hydroxide

This was prepared as the Cα salt according to the method of Bertho and Grassman (1). In spite of careful attention to detail, different preparations varied in their ability to adsorb heparin from plasma. However, once a suitable product was obtained it was stable for several months when stored at a temperature of 2–4 degrees Centigrade. Each milliliter of plasma was treated with 0.15 ml. of the aluminum hydroxide. Adsorption was carried out at room temperature for a period of 15 min. with an occasional mixing during that time.

Thromboplastin Solutions

A full strength (F.S.) solution was prepared by extracting one ampoule of the Difco rabbit brain material with 5 ml. of physiological saline at 50° C. for 10 min. From this, dilute solutions were prepared as required and expressed as a fraction of the full strength solution.

Clotting Times

(*a*) *Whole Blood Clotting Times:* tests were carried out by a modified Lee and White method, two tubes being used for each determination.

(*b*) *Recalcified Clotting Times:* tests were carried out by adding 0.1 ml. of a 0.025 *M* solution of calcium chloride to 0.2 ml. of plasma.

(*c*) *Thrombin Clotting Times:* a concentrated solution containing 140 units of Parke Davis Topical Thrombin in 50% glycerol and 50% saline was used as a stock solution. For the test, dilutions of 1 : 10 and 1 : 20, and occasionally 1 : 40 were used.

To 0.2 ml. of plasma, 0.1 ml. of saline and then 0.1 ml. of the thrombin solution were added. The 0.1 ml. of saline was introduced as a medium in which to add heparin or other reagents to the system.

Papain

The papain used was a product manufactured by the Nutritional Biochemical Corporation Laboratories at Cleveland, Ohio, for chemical and investigational use only. It was given intravenously as a saline suspension. Rabbits were given 60–100 mgm. in 5 ml. and dogs 350–400 mgm. in 20 ml. The injection was carried out rapidly, varying in time from 20 to 45 sec.

Results

In Table I are shown typical results of adding varying concentrations of papain and trypsin to 0.4 ml. quantities of citrated blood. In all of our *in vitro* tests the addition of 0.1 ml. of 0.025 *M* calcium chloride greatly enhanced the coagulation properties of both papain and trypsin. Indeed it was only with the more concentrated solutions of papain that clotting took place at all in citrated blood without the addition of calcium chloride.

TABLE I

THE INFLUENCE OF PAPAIN AND TRYPSIN ON THE CLOTTING TIME OF CITRATED RABBIT'S BLOOD, BOTH WITH AND WITHOUT THE ADDITION OF CALCIUM CHLORIDE

Amount of enzyme added, mgm.	Clotting time, sec.			
	Papain		#	Trypsin
1.00	35	300*	17	135*
0.50	43	>600*	23	140*
0.25	115		25	300*
0.12	127		29	550*
0.06	240		35	>600*
0.03	270		55	
0.00	300		310	

* *Saline added in place of calcium chloride.*
\# *A 1 : 250 Difco standardized product.*

The *in vivo* action of papain is in direct contrast to its *in vitro* action. The blood of rabbits becomes completely incoagulable within a few minutes after the intravenous injection of 60–100 mgm. of papain. The results obtained from a group of 10 healthy male rabbits, ranging in weight from 2.5 to 3.0 kgm., are shown in Table II. All these animals survived the experiment. In every instance heparin could be detected in the blood within a few minutes after the injection. It often increased in concentration up to two hours and then gradually decreased. In most cases coagulation was still prolonged after 24 hr. As further proof that the extracted material was heparin, the following experiment was carried out. Four rabbits were injected with papain and one hour after the injection they were bled out and the plasma pooled. A total of 100 ml. of plasma was obtained and on treatment it yielded an extract containing 26 units of heparin by antithrombic titration. This

material showed metachromatic and anticoagulant potency in good agreement with that shown for the heparin extracted from rabbits in anaphylactic shock reported by Monkhouse *et al.* (8). This experiment was repeated, with similar results, on another group of four rabbits.

TABLE II

HEPARIN RELEASED IN RABBITS BY THE INTRAVENOUS INJECTION OF PAPAIN

Animal No.	Papain, mgm.	Heparin, units per ml. of blood			
		½ hr.	1 hr.	2 hr.	4 hr.
1	60		0.20	0.12	
39	60	0.30		0.22	0.20
	75	0.30	0.30		
48	75	0.27	0.25	0.12	
50	75	0.24	0.10		
52	75	0.10	0.12	0.12	0.15
	90		0.20	0.20	
55	60		0.05	0.10	0.06
90	100	0.02	0.08	0.08	
86	75	0.12	0.18		0.18
85	75	0.04	0.10	0.10	
89	60	0.07		0.14	

In Table III are shown typical results of an intravenous injection of 400 mgm. of papain in a 10 kgm. dog. As in the experiments on rabbits, it was found that heparin was released in every instance with doses of this order. With the exception of one animal, which suffered profound shock, the amounts released were only about 1/10th those found in the blood of dogs suffering severe anaphylactic or peptone shock. This is in contrast to the rabbits, which, if anything, released more heparin after papain injection than during anaphylactic shock.

TABLE III

EFFECT OF AN INTRAVENOUS INJECTION OF 400 MGM. OF PAPAIN IN THE DOG

Time after injection, min.	Thrombin Clotting Times, sec.				Heparin, units/ml. blood
	Before $Al(OH)_3$		After $Al(OH)_3$		
	1 : 10	1 : 20	1 : 10	1 : 20	
0.0	17	30	22	34	0.01
15.0	210	*	24	36	0.35
90.0	93	213	22	35	0.18
150.0	35	94	23	36	0.07
320.0	33	52	22	37	0.02

* *Greater than 300 sec.*

Confirmation of the heparin titer is shown by the thrombin clotting times which vary in direct proportion to the amount of heparin extracted. Furthermore these times could be restored to normal by treating the plasma with aluminum hydroxide. This treatment has been shown to remove heparin from plasma (7). As will be shown later there is not always such a good agreement between heparin titer and the Thrombin Clotting Times.

In some experiments the Recalcified Clotting Times of citrated plasma taken after a papain injection were considerably shortened as compared to simultaneously taken Whole Blood Clotting Times. This was by no means a constant finding. However, addition of calcium chloride always resulted in a marked shortening of the Thrombin Clotting Times of these samples.

The influence of calcium chloride on the Thrombin Clotting Times is illustrated in Fig. 1. This animal received 400 mgm. of papain intravenously. It is clearly shown that calcium chloride caused a marked shortening of the Thrombin Clotting Times. The Whole Blood Clotting Times of all samples taken after the papain injection were greater than six hours. The Recalcified Clotting Times of all samples except the one taken at the 290 min. period were also greater than six hours.

This shortening of the Thrombin Clotting Times can be observed also when coagulation is prolonged by the injection of commercial beef heparin, provided of course the amount of heparin injected is not greatly in excess of one unit per ml. of plasma. Fig. 2 illustrates the effect of added calcium chloride on the Thrombin Clotting Times of citrated dog plasma at various times after the animal had received an intramuscular injection of 8000 units of heparin. Note the strong inhibitory action of calcium chloride on the antithrombic potency of heparin. The similarity of the results of these two

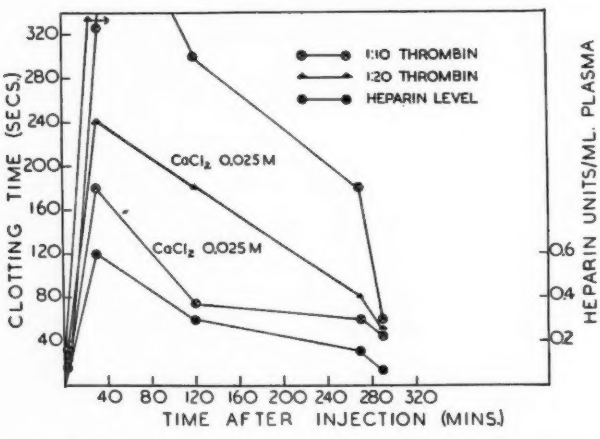

FIG. 1. The effect of calcium chloride on the Thrombin Clotting Time of plasma from a dog which had previously received an intravenous dose of 400 mgm. of papain. → Clotting time greater than 600 sec.

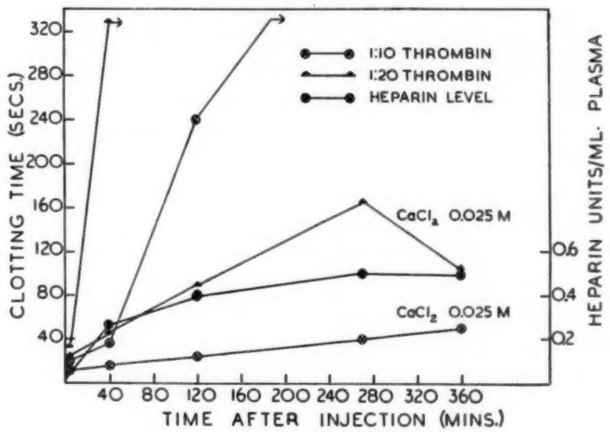

FIG. 2. The effect of calcium chloride on the Thrombin Clotting Times of plasma from a dog which had previously received an intramuscular injection of 8000 units (0.8 ml.) of beef heparin. → Clotting time greater than 600 sec.

experiments provides further evidence that the presence of heparin in the blood accounts for at least part of the prolonged clotting time which follows an injection of papain.

Jacobsen and Plum (5) showed that calcium ions inhibited the anticoagulant effect of heparin, but did not carry their experiments further than this. Our experiments indicate that calcium chloride interferes with the antithrombic power of heparin. Indeed it may be that calcium chloride exerts its main action at this phase of clotting since the Thrombin Clotting Times of blood heparinized to a moderate degree could be brought much nearer normal with calcium chloride than could the Recalcified Clotting Times.

Further studies comparing the anticoagulant effect of an injection of papain with the anticoagulant action of commercial beef heparin were made on rabbits. The results of a typical experiment are shown in Table IV. Two samples of blood were taken prior to the injection of papain, one into one-ninth its volume of citrate and the other into one-ninth its volume of saline containing enough heparin to give a final concentration of 0.5 units per ml. of blood. Ninety minutes after the animal had received an intravenous dose of 75 mgm. of papain, three more samples were taken, one into citrate, one into saline, and the third into a clean dry siliconed tube. Thrombin Clotting Times were carried out (in ordinary pyrex clotting tubes) on the plasma of all samples using varying amounts of both thrombin and calcium chloride. The extractable heparin in the papain sample assayed at 0.2 units per ml. of blood, which is a concentration less than half that added to the normal sample. The coagulability, however, was affected to a much greater extent in the papain sample.

TABLE IV

A COMPARISON OF THE EFFECTS OF VARYING CONCENTRATIONS OF CALCIUM CHLORIDE AND THROMBIN ON THE THROMBIN CLOTTING TIME OF HEPARINIZED RABBIT PLASMA AND PLASMA OBTAINED AFTER AN INTRAVENOUS INJECTION OF PAPAIN

		Thrombin Clotting Times (sec.)				
		Normal plasma		Papain plasma		
Thrombin, % F.S.	$CaCl_2$, *M*	Citrate	Heparin and saline	Citrate	Saline	Silicone
10	0.000	24	62	600	63	300 ±
"	0.025	20	35	240 ±	51	270 ±
"	0.050	16	27	63	56	240 ±
"	0.100	14	21	45	59	115
5	0.000	40	210 ±	600	138	600
"	0.025	25	120 ±	330	110	600
"	0.050	21	75	120	100	600
"	0.100	21	75	120	100	540 ±
2.5	0.000	60	600	600	180	600
"	0.025	31	600	600	130	600
"	0.050	26	240 ±	240	122	600
"	0.100	25	57	180	110	600

± *Partially clotted.*
F.S. thrombin is 140 units per ml.

The addition of calcium chloride greatly reduced the Thrombin Clotting Times of the normal sample taken into heparin and the papain sample taken into citrate but had only slight effect on the other two papain samples. In this regard the coagulation defect brought on by papain differs from an

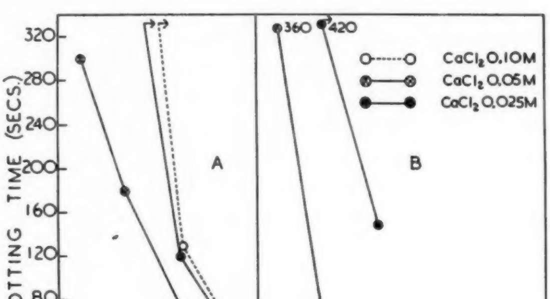

FIG. 3. The effect of calcium chloride on the Thrombin Clotting Times of plasma from a rabbit which had previously received an intravenous injection of 500 units of beef heparin. *A*—samples taken into citrate. *B*—samples taken into saline. → Clotting time greater than 600 sec. A 1 : 10 thrombin dilution was used.

intravenous injection of heparin. In the latter case the calcium chloride has a greater effect on the inhibiting action of heparin when the sample is taken into saline than when it is taken into citrate. A typical result of an intravenous injection of heparin is shown in Fig. 3. This rabbit received 500 units of heparin and duplicate samples of blood were withdrawn at intervals thereafter. One sample was taken into citrate and the other into saline. It can be seen from the graph that calcium chloride lowered the Thrombin Clotting Times of the sample taken into saline to a greater degree than those taken into citrate. It is difficult to understand why this should be so. This experiment has been repeated however, with similar results.

Another point brought out in Table IV is that the Thrombin Clotting Times of the sample taken into saline were much shorter than those of the samples taken into the dry siliconed tube. This difference appeared to be due to the diluting effect of the saline.

Tocantins (11) has shown that dilution with saline shortens the clotting time of both normal and hemophilic plasma. We found that blood or plasma made incoagulable by an injection of papain often clotted when diluted 30% with saline. This result appeared to be due mainly to an effect on the thrombin fibrinogen reaction, since dilution had a greater relative effect on the Thrombin Clotting Time than on the Whole Blood Clotting Time.

Fig. 4 illustrates the results from a typical experiment in which varying concentrations of thromboplastin were added to rabbit plasma and the

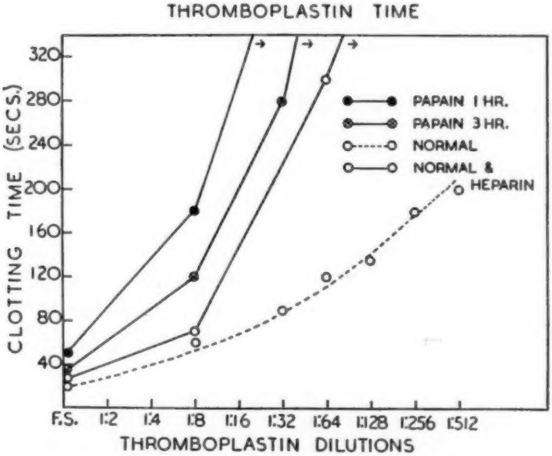

FIG. 4. The effect of adding varying concentrations of thromboplastin to the Recalcified Clotting Times of rabbit plasma. A comparison is made of normal and heparinized plasma and plasma after an intravenous injection of 75 mgm. of papain. → Clotting time greater than 600 sec.

Recalcified Clotting Times measured. The graph compares normal plasma, heparinized plasma, and plasma obtained after an injection of 75 mgm. of papain. Both samples of plasma taken after papain injection responded, to dilute solutions of thromboplastin, in a manner similar to heparinized plasma. On the other hand, while the concentrated solutions reduced the clotting time to the normal in the heparinized samples, they failed to do so in the samples taken after injection of papain. It should be pointed out that the concentration of extractable heparin in the papain plasma was less than half of that in the heparinized plasma.

Thus again we observe that the effect of papain cannot be explained entirely on the basis of extractable heparin. The results shown in Fig. 4 indicate that the additional disturbance is not due to a deficiency of thromboplastin.

Conclusion

When papain is injected intravenously into rabbits or dogs their blood becomes incoagulable. This effect is most pronounced in the rabbit, persisting for many hours.

Heparin is released into the blood in all instances but the amount extractable does not satisfactorily account for all the anticoagulant effect.

As already mentioned, Kellner *et al.* (6) suggested that the defect resembled hemophilia. From the results of our experiments we can not agree with these authors, but are of the opinion that the defect is in the thrombin–fibrinogen reaction. We have been unable to show any quantitative change in the fibrinogen and no evidence of fibrinolysis. There may however be a change in the quality of the fibrinogen which renders it less reactive to thrombin. There also remains the possibility of an increase in one of the other antithrombins as described by Seegers *et al.* (10).

In two of the rabbits surviving for some time after the injection of papain there was evidence of a mild disturbance in the coagulation system lasting for as long as two weeks. In the light of this finding it may be of interest to investigate this problem further using repeated small doses of papain or slow injections over a prolonged period.

An interesting side issue which arose out of the experimental work on papain was the inhibiting action of calcium chloride on the anticoagulant action of heparin. This effect of calcium chloride appeared to be most powerful at the thrombin fibrinogen phase.

Acknowledgments

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EMBRYONIC AND POSTEMBRYONIC CHANGES IN THE LIPIDS OF *ASCARIS LUMBRICOIDES* EGGS[1]

BY DONALD FAIRBAIRN

Abstract

Unembryonated ascaris eggs contained amounts of saponifiable and unsaponifiable lipids estimated to exceed 50% of the protoplasmic solids. The saponifiables (75–80% of the total lipids) consisted of triglycerides and much smaller quantities of phospholipids. Volatile acids comprised a significant part of the total triglyceride fatty acids. The unsaponifiables (> 20% of the total lipids) contained ascaryl alcohol as the major component. Unesterified sterols were also present. During embryonation and postembryonic survival of the eggs, the saponifiables decreased to 25% of the initial amount present, whereas unsaponifiables remained constant. Death of the embryos coincided with failure to utilize the remaining saponifiables.

Introduction

The very numerous eggs (2) of *Ascaris lumbricoides*, an intestinal roundworm occurring in pigs and humans, are passed from the host in the faeces, and under favorable conditions embryonate on the ground. There they may remain alive for a long time. Ascaris eggs, like those of many other nematodes, have a tough, complex, and highly impermeable shell (1, 11) and can be assumed to be cleidoic. They must, therefore, be self-contained in respect of nutriment, and by analogy with other cleidoic eggs, would be expected to contain, and to utilize, large amounts of fats (12). Flury found, in a classical investigation (7), that the female reproductive organs were rich in these substances, some of which were of an unusual nature. This work was recently confirmed and extended (4) in an investigation which reaffirmed the presence of a large amount of ascaryl alcohol, and established the presence of esterified volatile acids and of a complex sterol mixture.

The present report is concerned with the lipids of fertilized, but unembryonated eggs, and with some of the changes which occur during and following embryonation.

Methods

Living specimens of ascaris were collected at the slaughter house and maintained in the laboratory as previously described (4). Eggs were obtained by removing the paired uteri from females and expressing the contents of the terminal (vaginal) halves on to a glass plate. This material (about 0.3 gm. per worm and representing some 30% of the weight of the total reproductive system) was pooled and mixed. If not used immediately it was stored for a few days at 2° C. at which temperature no development of the egg occurred.

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The mixture is referred to subsequently as *uterine eggs*, and contained the eggs, covered with a characteristic sticky coat, and other uterine secretions of unknown nature.

Since uterine eggs were unsuitable for experimentation, *decoated* eggs were prepared from them as follows. Two or 10 gm. portions of uterine eggs were transferred to centrifuge tubes and mixed well with three to four volumes of 0.5 *N* sodium hydroxide solution. This treatment was repeated once or more, until the eggs settled freely and without clump formation, the dissolved sticky coat and secretions being discarded. These *decoated* eggs could be stored for long periods at 2° C. without appreciable loss of viability.

Decoated eggs obtained from 2 or 10 gm. samples of uterine eggs were embryonated in 50 or 250 ml. cotton-stoppered Erlenmeyer flasks containing approximately 5 or 25 ml. of 0.1 *N* sodium hydroxide solution, respectively. In this dilute alkali suspension microbial growth was inhibited. The flasks were shaken continuously in a waterbath maintained at 30–31° C. during the embryonation period (10 days) or longer, as required. When the volume of the suspension and the rate of shaking were properly adjusted, all the eggs were in motion in the flask, and adequate oxygenation was assured.

Dry weights of uterine and washed decoated eggs were obtained by heating at 110° C. Wet weights of decoated eggs could be obtained approximately as follows. An aqueous suspension of decoated eggs was filtered at the pump on a medium porosity glass filter, following which the wet egg residue was washed quickly with a little ethanol (twice) and ether (twice). The residue was dried for three minutes at the pump, and for 30 min. in a 37° C. air incubator, before weighing. In this method, the surface drying with ethanol and ether resulted in the extraction from the egg of a little lipid material (about 2% of the total wet weight) and possibly of a little water. The eggs remained completely viable, however, and it was considered that the weight obtained was a good approximation to the true decoated wet weight.

Lipids were obtained from embryonated or unembryonated decoated eggs by overnight extraction at room temperature with 30 volumes of chloroform–methanol (2 : 1 v/v). The egg residue was re-extracted with solvent for a few hours, and the combined extracts were washed with water, taken to dryness, and the lipid residue redissolved in solvent (8).

The method used previously for the separation of saponifiable and unsaponifiable lipid fractions (4) was not successful when applied to small quantities of material, and as a result a more arduous procedure was used. Lipids equivalent to those occurring in 2 gm. of uterine eggs were saponified by refluxing for 30 min. in 2 ml. of *N* sodium ethoxide. The solution was made slightly acid by addition of 10 *N* sulphuric acid, and then neutralized with 0.1 *N* sodium hydroxide, using phenol red as external indicator. It was taken to complete dryness *in vacuo*, and the residue extracted twice with dry ether. The unsaponifiable extract so obtained was washed once with a little water, and the water added to the ether-insoluble residue, from which the saponifiable fraction could be obtained by conventional methods.

Phospholipids, ascaryl alcohol, and volatile and non-volatile acids were determined as previously described (4). Free and esterified sterols were estimated colorimetrically (13).

Results

The wet weight of unembryonated decoated eggs was 19.7% that of uterine eggs, a low value attributable to the higher water content of the latter (85%), and to the large amount of solids (31% of the total) occurring in the external egg coat and in other uterine secretions. Decoated eggs, on the other hand, contained 53% solids. Of these, the shell accounted for 35% and the protoplasm for 65%[2]. Thus, a simple calculation revealed that the protoplasm contained 45% of the total solids occurring in the uterine eggs, and the shell 24%. These mean values were reproducible within ± 5%, owing undoubtedly to the fact that the data for each experiment were obtained by analysis of pooled eggs representing many individual females.

Total lipids of embryonated and unembryonated decoated eggs were determined in a series of experiments, using material derived from 2 gm. initial weight of uterine eggs. The above data were then employed in calculating the lipid content of decoated eggs in terms of dry weight of uterine eggs, decoated eggs, and egg protoplasm (Table I). During embryonation the average decrease in decoated egg solids was 20%, and in lipids, 17%. Thus, the lipid concentration during embryonation remained essentially constant, and the embryo, as well as the egg, contained very large amounts of reserve fats. If these fats were assumed to occur only in the protoplasm, and not in the shell, they accounted for one-half the protoplasm dry weight in unembryonated eggs, and for somewhat more than this in embryonated eggs. This assumption is not entirely warranted (5), but it is clear that the lipids are present in remarkably high concentration.

TABLE I

TOTAL LIPIDS IN DECOATED EGGS

	Lipid concentration as % of total solids in		
	Uterine eggs (± standard deviation)	Decoated eggs	Egg protoplasm
Unembryonated*	23 ± 0.8	33	51
Embryonated†	19 ± 0.5	34	62‡

* *Average of five experiments.*
† *Average of five experiments. The total solids of decoated eggs decreased 20% during embryonation.*
‡ *Calculated by assuming that the decrease in solids during embryonation was confined to the protoplasm, the shell weight remaining constant.*

[2] *The separation of shell from protoplasm in unembryonated eggs was carried out by R. F. Passey, using an unpublished method which cannot be applied to embryonated eggs.*

The general procedure (4) devised for the fractionation of the lipids of the entire female reproductive system, except for the modification in the method of separation of saponifiables and unsaponifiables, was applied to the lipids of decoated eggs. In Table II the results of two such experiments, in good agreement, are summarized. In these experiments the eggs were incubated for 14 days, i.e., about four days more than required for embryonation, and consequently the differences between these eggs and embryonated samples were somewhat accentuated. The most striking feature of the results was the evident failure of the large unsaponifiable fraction to change in amount during embryonation, an observation which was confirmed in all subsequent experiments. The 24% over-all decrease in lipids, therefore, was attributable to decreases in trigylceride fatty acids, both volatile and non-volatile, amounting to 46 and 30%, respectively. Volatile acids represented a considerably smaller proportion of the total lipids than they did in the entire reproductive system (4), whereas non-volatile acids, unsaponifiables, and phospholipids were not greatly different.

TABLE II

LIPIDS OF EMBRYONATED AND UNEMBRYONATED DECOATED EGGS*

	% unembryonated decoated egg solids				% total lipids (mean value)	
Fraction	Unembryonated		Embryonated		Unembryonated	Embryonated
Total	36	32	26	26	100	100
Volatile acids	2.5	2.7	1.4	1.4	7.9	5.8
Non-volatile acids	19	21	13	15	57	58
Unsaponifiables	7.8	7.6	7.5	7.3	22	28
Phospholipids	3.1	3.3	2.8		9.2	11

* *Two experiments. Eggs embryonated 14 days at 31° C.*

During embryonation phospholipids decreased little, if any, in amount. Their properties were similar to those of the entire reproductive system. Analysis (%) showed, for unembryonated eggs, N 1.72, P 3.57; N:P (molar) 1.08; and for embryonated eggs N 1.72, P 3.70; N:P (molar) 1.04. Such analyses are typical for lecithins and cephalins, but not for sphingomyelins.

In decoated eggs, as in the entire reproductive system, ascaryl alcohol was the main component of the unsaponifiable fraction. It was isolated by taking advantage of its insolubility in ether, as previously described. Sterols were also present, as shown by precipitation with digitonin from the unsaponifiable fraction and subsequent colorimetric determination (13). The amounts obtained from the relatively small egg samples were insufficient to establish their nature, which in the reproductive system was complex (4). Although

the colorimetric factor previously determined was applied in calculating the values shown in the last column of Table III, the accuracy of this factor for eggs alone was not established. The data in this table show clearly that sterols remained constant in amount during embryonation; and since direct determination on a lipid extract without preliminary saponification yielded essentially the same results, it was concluded that sterol esters were not present in the eggs.

TABLE III

STEROLS IN DECOATED EGGS

	Total sterols	Free sterols	Calculated total*
	% unembryonated decoated egg solids		
Unembryonated	0.39	0.37	0.62
Embryonated	0.38	0.38	0.60

* *Color reaction of ascaris sterols relative to cholesterol = 0.63 (see the text).*

The results of a detailed analysis of the volatile fatty acids present in the saponified fats are given in Table IV. In unembryonated eggs, acetic, butyric, and pentanoic acids predominated. During embryonation the large (44%) decrease in volatile acids arose chiefly from decreases in pentanoic and butyric acids, and to some extent in acetic acid. No change was observed in hexanoic acids. The relative proportions of volatile acids in decoated eggs differed from those observed in the whole reproductive tissue (4), particularly as regards hexanoic and butyric acids.

TABLE IV

VOLATILE ACIDS OF DECOATED EGGS

(Millimoles per 100 gm. of unembryonated egg solids)

Fraction	Unembryonated eggs	Embryonated eggs	% utilization during embryonation
Acetic	9.8	7.6	22
Propionic	1.2	0.5	58
Butyric	5.8	3.2	45
Pentanoic	12.3	4.2	66
Hexanoic	1.7	1.7	0
Total	30.8	17.2	44

Ascaris eggs will survive for a long time if given favorable conditions, and it seemed likely that the large fat reserves of the embryo (Table II) would be utilized during this period. Accordingly, an experiment was undertaken in which the decoated eggs derived from 2 gm. samples of uterine eggs were withdrawn from the incubating bath at intervals, and analyzed for total fats and for unsaponifiables. Saponifiable matter was estimated by difference.

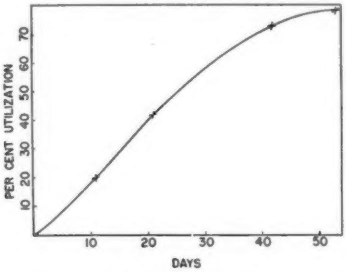

FIG. 1. Utilization of saponifiable lipids of ascaris eggs during embryonation and postembryonic survival at 31° C.

In this experiment, which was concluded after 53 days, the unsaponifiables remained constant at 8.1 ± 0.2% of the unembryonated decoated egg solids. Saponifiables, on the contrary, decreased rapidly during and following embryonation until after 42 days they comprised only 27% of the original amount (Fig. 1). Meanwhile the embryos, originally dark and opaque, had become almost transparent in appearance. During the next nine days the saponifiables decreased slightly, to 24% and many of the embryos showed unmistakable signs of disorganization, or of complete disintegration. Thus, deterioration and death appeared to coincide with exhaustion of available fat reserves.

Discussion

Ascaris eggs are probably fertilized by the highly specialized spermatozoa (6) while they are still in the primary oöcyte stage. Fertilization initiates processes which result in a striking decrease in egg volume, two reduction divisions of the egg nucleus with rejection of the polar bodies from the protoplasm, union of the two pronuclei (egg and sperm), and formation of the complex shell (6). These events occur in the oviduct and upper regions of the uterus, and concurrently a thick and sticky coat is laid over the shell by means of uterine secretions. The biochemistry of shell formation in particular, which involves the synthesis of proteins and of chitin from materials laid down in the oöcyte for this purpose, must be complex and extensive. It was for this reason that eggs from the upper half of the uterus were not used in the present investigation. The thick-shelled eggs in the lower half were about 95% fertile, and embryonated quite uniformly under the chosen conditions.

Although the energy requirements of the adult ascaris appear to be furnished primarily by means of a complex fermentation (9), oxygen is necessary for

embryonation of the mature eggs, and must be supplied at a lower temperature than that of the host. Adequate oxygenation of an egg suspension maintained at 30°–31° C. provided near-optimal conditions for development. Assurance that the lipids of eggs alone were being studied was provided by a preliminary treatment designed to remove the sticky coat and other uterine secretions.

From the results it is clear that ascaris eggs are extremely rich in fats, which are sufficient in amount not only to provide a concentrated source of nourishment for the embryo developing in the (presumably) cleidoic eggs, but to furnish an energy reserve adequate to preserve life for some time thereafter. This reserve is of obvious importance when it is realized that the embryonated egg must normally await ingestion by a suitable host before it can hatch and undergo further development. The fats so utilized occur exclusively in the saponifiable fraction, and are presumably triglycerides. It is notable that they include a considerable amount of the lower volatile acids, ranging from acetic to hexanoic, which, as pointed out previously (4), resemble closely the acids which the adult female excretes as waste fermentation products. It seems probable, therefore, that the economy of the parasite is so regulated that some of the energetically wasteful fermentation acids are incorporated into the ovarian triglycerides instead of being excreted, and are subsequently utilized during the oxidative metabolism of the developing egg. Differences in the amounts and relative proportions of volatile acids observed in triglycerides of the whole reproductive system (4) and of the eggs alone are not readily explained unless consideration is given to egg metabolism in the upper uterus, to which reference has been made, and concerning which information is not available.

Saponifiable lipids, including phospholipids, remaining at the time when death of the embryos occurred represented about 25% of the amounts originally present. This lipid residue might, by analogy with mammalian physiology (10), be of such nature or distribution as to be unavailable for purposes of energy metabolism. It may be, however, that the causes of death were not closely associated with fat metabolism.

The constancy of the unsaponifiable fraction during embryonation and subsquent survival of the egg suggests the existence of specific functions for its components distinct from considerations of energy metabolism. Cholesterol in the hen's egg remains unchanged during embryonation (3), as do the ascaris sterols; but in the former, cholesterol is a major component of the unsaponifiables, whereas the ascaris sterols represent a tiny part of the total unsaponifiables. Here ascaryl alcohol is the major component, and it is now known (5) that the greater part of this substance is present, not in the protoplasm of the egg, but in the extraordinarily tough and impermeable vitelline membrane which is formed soon after fertilization has taken place.

Acknowledgment

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THE LIPID COMPONENTS IN THE VITELLINE MEMBRANE OF *ASCARIS LUMBRICOIDES* EGGS[1]

BY DONALD FAIRBAIRN AND BEVERLEY I. PASSEY

Abstract

The preparation of embryonated ascaris eggs enclosed only by the vitelline membrane is described. No impairment of membrane structure or function was observed. Membrane and larval fractions were separated, and their lipids analyzed. The membrane lipids were nearly all unsaponifiable, and consisted mainly of ascaryl alcohol. Larval lipids were primarily saponifiable, but also contained significant amounts of ascaryl alcohol, and nearly all of the sterols. Because the vitelline membrane dissolved in chloroform and similar solvents, ascaryl alcohol was judged to be a major component. The results are compared with previous observations and hypotheses concerning the vitelline membrane of nematode eggs.

Introduction

The striking resistance of *Ascaris lumbricoides* eggs to many chemicals (8, 13) has been attributed generally to the unusual properties of the highly impermeable innermost, or vitelline, membrane. Although these and other nematode eggs remain unaffected by many salts, oxidizing and reducing agents, and acids and bases, they are readily destroyed by certain organic solvents, which penetrate and dissolve the vitelline membrane, and by heat, which melts it at approximately 70° C. Such properties, when considered in conjunction with histological observations made by Fauré-Fremiet (6) have led to the belief that the vitelline membrane of ascarids is essentially lipid in nature (3, 9, 11, 12, 14).

Fauré-Fremiet discovered ascaryl alcohol, an unusual and still chemically unidentified lipid, in the reproductive tissues of *Ascaris megalocephala*, and believed it to be the main component of the membrane. Chitwood (3), on the other hand, suggested without positive evidence that sterols were more probable components, while Timm (11) asserted that the membrane consisted of myricyl palmitate, which is also a major component of beeswax.

The lipids of ascaris reproductive tissues were recently reinvestigated in this laboratory (4) with results which confirmed the presence of large amounts of ascaryl alcohol and small amounts of a complex sterol mixture. Subsequently, it was found (5) that unsaponifiable lipids such as ascaryl alcohol and the sterols did not participate in the extensive utilization of fats which occurred during and after embryonation of the egg. This observation suggested a specific function for these substances which was independent of oxidative metabolism, and which might be identified with their presence in the vitelline membrane. The concentration of sterols, however, was too small to account for any large part of the membrane, so that ascaryl alcohol seemed to be the more likely component. By using a sequence of chemical and bacterial

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treatments it was possible to remove the outer egg shell, leaving the embryo encased only in the vitelline membrane, and then by a suitable method to remove this membrane for chemical analysis. This was done, with results which will be described.

Methods

Ascaris eggs were embryonated as described previously (5), with the exception that aseptic techniques were employed. They were then washed by centrifugation, after which most of the hard parts of the shell were dissolved by treating the eggs for 24 hr. at 40° C. with a solution containing 4 ml. of reagent grade sodium hypochlorite solution (5–6% available chlorine) per 100 ml. of N sodium hydroxide. Although this treatment occasionally dissolved all parts of the shell except the vitelline membrane, more often a very thin residual shell remained, which burst upon heating to 70° C., liberating larvae and melted membrane. The removal of this (presumptive) chitin residue from unheated eggs, while leaving the vitelline membrane intact, was accomplished by shaking the washed, hypochlorite-treated eggs for 48 hr. at 31° C. in a mineral medium containing chitinolytic bacteria. These bacteria had been isolated previously from pig gut contents in the chitin enrichment medium described by Benton (1). The eggs, washed free of bacteria, were now hatched almost completely by heating to 75° C. Upon cooling and centrifuging, the dead larvae formed a distinct dark layer overlying which was a white layer composed largely of globules derived from the membrane. This globular material was dispersed into the supernatant fluid by careful mixing, and then aspirated away from the larvae. A single resuspension and centrifugation of the larval layer yielded more globules which were combined with the first lot. The larval layer now contained a residue of globules estimated to be about 10% of the total. The membrane moiety, on the other hand, contained, besides 90% of the globules, most of the unhatched eggs (about five per cent), a very few larvae, all the substances (presently unknown) normally present in the extralarval egg fluids, and larval extractives, if any. These inaccuracies in fractionation were considered to be satisfactorily small. Losses of eggs during the several manipulations involved in treatment and fractionation were unavoidable but did not exceed an estimated 10%.

Chemical analyses were performed by highly reproducible procedures specifically designed for ascaris eggs (4, 5). Total lipids were saponified and the saponifiable and unsaponifiable substances isolated and weighed on a semimicrobalance. Ascaryl alcohol was isolated from the unsaponifiable fraction semiquantitatively on the basis of its insolubility in ether, recrystallized once, and identified by melting point, and mixed melting point with authentic ascaryl alcohol obtained from large batches of reproductive tissue (4). Sterols were precipitated from the unsaponifiable fraction by addition of digitonin, and determined colorimetrically (10), using cholesterol as a standard. The observed results were divided by the factor 0.63 to obtain the value for total sterols (4).

Results

The chemical and bacterial degradation of the hard parts of the ascaris egg-shell were consistently reproducible. Although the appearance of the vitelline membrane was unaltered by such treatment, this criterion alone was not considered to be sufficiently rigorous. Additional, and more conclusive, evidence that the membrane remained, in fact, unaffected was afforded by exposing such eggs, and untreated controls, to representative solutions for 24 hr. Hydrochloric acid (2 *N*), nitric acid (2 *N*), sodium hydroxide (2 *N*), ammonium hydroxide (0.5 *N*), formaldehyde (3.3 *N*), and sodium chloride (4 *N*) failed to penetrate the membrane, inside which the embryos remained alive and motile. If, however, the treated eggs were hatched by light crushing under a cover slip, the hatched larvae were killed quickly by all these solutions. Organic solvents such as chloroform dissolved the membrane and killed the larvae, whereas heat melted it. These results provided strong evidence that the vitelline membrane was unimpaired by the procedures employed in removing the hard shell, and furnished in addition direct proof that, as long suspected, this membrane is primarily responsible for the remarkable toughness and impermeability of ascaris eggs.

Two experiments were made in which the larval and membrane moieties were examined chemically. In each, 10 gm. of uterine eggs were decoated and embryonated for 28 days at 31° C. The hard shell was then removed as described and the egg residue suspended in 5 ml. of water in a 50 ml. Erlenmeyer flask. This was immersed in a water bath at 83° C. until the temperature of the suspension rose to 75° C. (one minute). The flask was cooled immediately in ice, the suspension transferred to a 12 ml. centrifuge tube, and the hatched larvae separated from the membrane moiety. The two fractions were dried *in vacuo* at room temperature, weighed, and the lipids extracted and analyzed. The white, fat-free membrane residue was unexpectedly large, and nearly all water-soluble. Probably, therefore, it originated in the extra larval egg fluids, which though abundant are of unknown nature, or, in part, as water-soluble extractives from the larvae themselves. Under existing circumstances there was no method available for the investigation of these alternatives.

In the first of these experiments part of the egg suspension was lost, but the analytical data agreed proportionately with those of the second experiment, the results of which are summarized in Table I.

TABLE I

LIPIDS OF THE LARVAE AND THE VITELLINE MEMBRANE IN EMBRYONATED ASCARIS EGGS

(Mgm. per 10 gm. of uterine eggs)

	Dry weight	Fat-free weight	Lipids	Unsapon-ifiables	Sapon-ifiables	Ascaryl alcohol	Sterols
Membrane	193	109	59	53	3	41	0.1
Larvae	240	167	82	19	60	11	1.3

It is apparent from this table that there were distinct differences in the lipids extracted from the two egg fractions. The membrane moiety contained a considerable amount of lipids, nearly all of which were unsaponifiable. By solvent fractionation ascaryl alcohol was found to account for 77% of these unsaponifiables, a value which can be regarded as minimal. Sterols, on the contrary, were virtually absent, and the small amounts present certainly originated in part in the unhatched egg impurity. In contrast, the larval lipids contained nearly all the saponifiable matter (fatty acids arising chiefly from neutral fats and phospholipids) and sterols. In addition, they contained significant amounts of ascaryl alcohol, some of which no doubt originated in the membrane impurity occurring with this fraction.

Mention may be made of the apparently low total weight of larval and membrane moieties. Ten grams wet weight of uterine eggs provide, on the average, 1.05 gm. dry weight of unembryonated eggs (5). This includes the hard shell, conservatively estimated as 30% of the total, or 0.315 gm., which, in these experiments, was removed by digestion. During a 10-day embryonation period, furthermore, there is a decrease in egg weight of some 20%, or 0.210 gm. Thus, the calculated dry weight of treated eggs is 0.525 gm. and the observed weight found by adding the membrane and larval weights was 0.433 gm. This is a good agreement, if allowance is made for losses of eggs which occurred during the numerous manipulations, and for weight decrease occurring between the 11th and 28th day of incubation.

In the membrane moiety the sum of the lipid and fat-free residue weights accounted for only 87% of the initial dry weight. Apparently low recoveries such as this arise from unweighed losses of non-lipid impurities during washing of the original lipid extracts with water (7), or from the protein components of proteolipids. In the present experiment the unpurified (unwashed) extract weighed 82 mgm., whereas the purified extract (Table I) weighed 59 mgm.

Discussion

There can be little doubt, for reasons previously stated, that ascaris eggs treated as described lost all other membranes and retained the vitelline membrane intact. The use of heat to melt the membrane and liberate the embryos is objectionable in that the embryo is killed, and as a result the permeability of its integument may be altered sufficiently to permit extraction of certain substances into the membrane moiety. Such substances, however, would be expected to be water soluble, and thus would not affect the results of lipid analyses. Nevertheless, unsuccessful attempts were made to secure quantitative hatching of relatively large samples of treated eggs by other methods. Alternatively, an effort was made to secure cultures of microorganisms which would digest the viteliine membrane, for in this way the composition of the membrane could be inferred by comparing the composition of treated eggs (with membrane) and larvae. Such cultures were obtained, but unfortunately could not be applied successfully or reproducibly to large samples of eggs; or, in some cases, they digested the embryo as well as the vitelline membrane.

Strong evidence that the use of heat for hatching was not open to serious objection in the present investigation was furnished by the results of the lipid analyses, which demonstrated clearly that under the conditions used the larval lipids remained strikingly different in composition from the membrane lipids. Thus, virtually all the sterols and saponifiables were found in the larvae, and three quarters of the unsaponifiables in the membrane moiety. Some, perhaps half, of the unsaponifiables, other than the sterols, found in the larvae were due to contamination with membrane globules. It is possible, however, that ascaryl alcohol, the major component of the unsaponifiable fraction, is not limited in distribution to the vitelline membrane, but may occur also in the cuticle or subcuticle of the embryo. Thus, von Brand and Winkeljohn (2) found ascaryl alcohol in male ascarids and Chitwood (3) postulated the existence of a lipid-containing membrane in larval and adult nematodes, as well as in the eggs.

A lipoidal vitelline membrane, similar in properties and function to that of ascaris, occurs in many nematode eggs (3, 9, 11, 14). Chitwood (3), and Jacobs and Jones (9), believed the major constituent to be a sterol or related substance, but presented no positive supporting evidence. Timm (11), on the other hand, mistakenly and from wholly inadequate evidence identified the membrane lipid of ascaris with myristyl palmitate. These assertions are all the less easy to understand when it is recalled that Fauré-Fremiet (6), after careful histological study, concluded that ascaryl alcohol was the major membrane lipid. Fauré-Fremiet's results have received complete chemical substantiation in the present investigation, and it is possible that ascaryl alcohol occurs generally in nematode eggs. It may be mentioned, however, that the vitelline membrane is undoubtedly complex in nature, with substances other than ascaryl alcohol contributing to its structure.

Acknowledgments

We are indebted to E. Meerovitch for a translation from the Russian of the cited work of M. M. Zawadowsky; and to R. P. Harpur and R. F. Passey for advice and assistance.

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INHIBITION OF CHOLESTEROL FORMATION BY RAT LIVER HOMOGENATES[1]

By B. B. Migicovsky

Abstract

The inability of liver homogenates, from starved and vitamin A deficient rats, to synthesize cholesterol is illustrated. A possible reason for this phenomenon is that these preparations inhibit cholesterol synthesis when added to a liver homogenate from a normal rat. The inhibitory factor or factors are present in both the supernate and residue portions of the homogenate, although the residue matter is more inhibitory.

The formation of cholesterol from acetate has been demonstrated repeatedly both *in vivo* and *in vitro*. Bucher (1) succeeded in preparing a homogenate of rat liver which was able to synthesize cholesterol, as measured by incorporation of C^{14}-acetate.

Hutchens *et al.* (2) showed that a fasting rat has a decreased ability to incorporate acetate into cholesterol and fatty acids. Other studies *in vitro* by Lyons *et al.* (3), Tomkins and Chaikoff (7), and Medes *et al.* (4) have also shown large decreases in lipogenesis after short fasting periods.

The phenomenon of a decreased lipogenesis came to our attention when we attempted to study cholesterol synthesis by livers from vitamin A deficient rats. The present study is the initial part of the investigation into the reasons for the decreased lipogenesis following a period of starvation. The possibility that the effect of a vitamin A deficiency is similar to the starvation effect is likely, since anorexia is quite severe in vitamin A deficiency.

Methods

All rats used in this study were from our own colony and were two to three months old when their livers were taken for experiment. Vitamin A deficient rats had been fed the deficient diet since weaning, and the starved rats had been without food for 48 hr.

The cholesterol synthesizing system used was described by Bucher (1). Cholesterol was isolated as the digitonide according to the method described by Rabinovitz and Greenberg (6). The digitonide was plated on paper by the method described by Migicovsky and Evans (5) and counted in a gas flow counter. Corrections for self-absorption were applied. Decomposition of the digitonide with pyridine and reprecipitation did not alter the specific activity.

Incubations were carried out in Warburg flasks, in an atmosphere of oxygen, for three hours at 37° C. Each flask contained 1.3 mgm. ATP, 3.8 mgm. DPN, 1.0 μmole C^{14}-acetate solution. The final volume of 2.4 ml. was made up with the same buffer mixture as was used for homogenization. Five milligrams of carrier cholesterol was added to each flask prior to saponification.

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Results are expressed as micromoles of acetate incorporated into the total cholesterol. This value is equivalent to $\frac{\text{counts per minute/mgm. digitonide}}{\text{counts per minute}/\mu\text{ mole acetate}} \times 20$. The factor of 20 is the cholesterol digitonide equivalent of 5 mgm. cholesterol.

Results and Conclusions

The data in Table I illustrate the inability of preparations from vitamin A deficient and starved rats to incorporate acetate into cholesterol. A possible reason for this is that small quantities of liver homogenate from starved and A deficient rats inhibit the reaction. This is shown in Table II which illustrates the inhibitory character of the above-mentioned preparations. It will be noted that the starved rat preparations are more inhibitory than those from vitamin A deficient rats.

TABLE I

CHOLESTEROL SYNTHESIS FROM C^{14}-ACETATE BY LIVER HOMOGENATES FROM NORMAL, VITAMIN A DEFICIENT, AND STARVED RATS

Exp. No.	Homogenate preparation	μM. acetate incorporated $\times 10^3$
28	2 ml. normal rat liver	12.3
	2 ml. vit. A deficient	0
	2 ml. vit. A deficient plus vit. A	0
34	2 ml. normal	14.4
	2 ml. vit. A deficient	0
	2 ml. vit. A deficient*	.03
49	1.5 ml. normal	8.8
	1.5 ml. starved	0
61	1.5 ml. normal	12.0
	1.5 ml. starved	0

* *Vitamin A was fed to the rats in the diet (500 units/gram) for one* week.

TABLE II

INHIBITION OF CHOLESTEROL SYNTHESIS BY LIVER HOMOGENATES OF VITAMIN A DEFICIENT AND STARVED RATS

Addition to 1.5 ml. of normal rat liver homogenate	μM. acetate incorporated $\times 10^3$		
	Trial No.		
	49	50	52
None	8.8	10.5	17.6
0.5 ml. starved homogenate	2.3	0.9	2.1
0.5 ml. vit. A deficient homogenate	4.5	3.4	—

TABLE III

INHIBITION OF CHOLESTEROL SYNTHESIS BY SUPERNATE* AND RESIDUE* OF CENTRIFUGED HOMOGENATES

Addition to 1.5 ml. of normal rat liver homogenate	μM. acetate incorporated × 10³		
	Trial No.		
	50	53	57
None	10.5	17.5	15.8
0.5 ml. normal supernate	7.4	16.4	12.1
0.5 ml. normal residue	8.5	11.0	10.2
0.5 ml. vit. A deficient supernate	8.5	—	—
0.5 ml. vit. A deficient residue	4.6	—	—
0.5 ml. starved supernate	3.6	12.4	5.4
0.5 ml. starved residue	2.6	5.2	3.5
0.5 ml. starved homogenate	—	8.2	2.0

* *An aliquot of the homogenate prepared according to Bucher (1) is again centrifuged in a Servall centrifuge at 13,500 r.p.m. for 20 min. The supernate is decanted and the residue is resuspended in buffer to the original volume of the aliquot.*

Initial attempts to localize the inhibitory factors are shown in Table III. In these experiments the homogenate was centrifuged in a refrigerated Servall centrifuge at 13,500 r.p.m. and separated into supernate and residue. The data indicate that normal supernate or residue frequently reduced the amount of acetate incorporated. The effect of the residue was usually greater than that of the supernate. The effect of preparations from the vitamin A deficient and starved animals was quite marked and the residue was always more inhibitory than the supernate.

The comparative effect of the residue from centrifuged liver homogenates of normal and starved rats is shown in Table IV. When the residue from

TABLE IV

EFFECT OF RESIDUE* ON CHOLESTEROL SYNTHESIS WHEN ADDED TO SUPERNATE* OF RAT LIVER HOMOGENATES

Liver preparation	μM. acetate incorporated × 10³	
	Trial No.	
	52	57
1.5 ml. normal homogenate	17.6	15.8
Addition to 1.5 ml. normal supernate		
None	0.33	4.51
0.05 ml. normal residue	5.50	—
0.10 ml. normal residue	—	6.03
0.15 ml. normal residue	5.90	—
0.25 ml. normal residue	7.2	6.40
0.50 ml. normal residue	6.6	8.76
0.05 ml. starved residue	0.78	—
0.10 ml. starved residue	—	1.70
0.15 ml. starved residue	0.16	—
0.25 ml. starved residue	—	1.16
0.50 ml. starved residue	—	0.34

* *Same as in Table III.*

normal rats was added to 'normal' supernate it always increased the degree of acetate incorporation. Addition of residue from starved rats to the supernate from normal animals decreased the degree of acetate incorporation progressively as the amount of residue was increased.

The results indicate that a reason for decreased lipogenesis in starved rats may be found in the presence of a factor or factors which interfere with cholesterol synthesis or degrade the cholesterol that is synthesized.

Acknowledgment

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TONIC ADAPTIONS IN DEAFFERENTED LIMBS OF THE CAT[1]

BY R. D. TEASDALL[2] AND G. W. STAVRAKY

Abstract

In 26 cats postural reflexes and tonic adjustments in the chronically deafferented and intact limbs were studied by means of moving picture and other photographic recordings for a period of four to six months. The positive supporting reactions which were dependent on an intrinsic reflex arc were permanently abolished by the deafferentation. Other segmental reflexes such as the crossed extensor and Philippson's reflex were readily elicited in the deafferented extremity within 24 to 48 hr. after section of the posterior nerve roots, and after longer intervals following the deafferentation these reflexes became greatly exaggerated. They appeared sooner after the operation and became more prominent in the hind limb than in the fore limb.

The intersegmental postural reflexes and the scratch reflex were consistently present in the deafferented limbs of chronic animals and the latter reflex became markedly hyperactive within one week after section of the posterior nerve roots. The normal reflex responses to linear acceleration were first depressed by section of the posterior nerve roots, but in one to four weeks after the operation they were readily demonstrable, became exaggerated in the deafferented extremity, and remained so for the rest of the period of observation. Unlike the crossed extensor reflex, these reflexes reappeared first in the fore limb and reached greater prominence in this extremity than they did in the hind limb. A reversal of the tonic labyrinthine and neck reflexes was demonstrated in the deafferented extremities of the chronic animal. This reversal appeared one to two months after section of the posterior nerve roots in the fore limb, and two to three months following deafferentation of the hind limb. When established, the reversal was readily elicited and took place simultaneously with a normal response in the intact extremities on the opposite side of the body. Simultaneously with the changes in postural reflexes, alterations in tone became prominent.

The modified and exaggerated postural reflexes and tonic adjustments observed in the deafferented limbs were attributed to a sensitization of the partially denervated spinal neurones to nerve impulses reaching them from various receptive fields other than those originating in the extremity itself. It is felt that the modifications in postural reflexes and subsequent alterations of muscle tone which have been demonstrated in the chronically deafferented extremities of animals may play an important part in the pathogenesis of human sensory ataxia.

Introduction

The difference between the normal and acutely deafferented quadriceps muscle in the decerebrate preparation was described by Sherrington (22). The intact muscle always developed decerebrate rigidity, showed an exaggerated tendon jerk, and exhibited prominent lengthening and shortening reactions. The acutely deafferented quadriceps revealed none of these stretch phenomena which Sherrington believed to be characteristic of the tonic state. Accordingly, the proprioceptive reflex theory of muscle tone was based on the fact that tone in decerebrate animals was primarily a stretch reflex dependent on the afferent nerve supply of the extensor muscles (10, 21, 24).

Although decerebrate rigidity was abolished by dorsal root section, the deafferented muscle was found by Sherrington (22) to participate in other postural adaptations such as the crossed extensor reflex. Furthermore,

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[2] *Postgraduate Medical Fellow, National Research Council of Canada.*

stimulation of suprasegmental areas of the central nervous system produced tonic adjustments in the deafferented quadriceps (15, 23). From these findings Magnus (13, 14) concluded that while tone of a given muscle depended essentially upon the integrity of the posterior nerve roots, it was not wholly independent of afferent impulses from other levels.

The purpose of this investigation was to study in detail these intrinsic postural reflexes and tonic adjustments in the chronically deafferented limbs of the cat.

Method

Deafferentation of one limb was carried out in 26 cats. The fore limb was deafferented in 14 animals by means of aseptic intradural section of C_3 to T_3 dorsal roots. A similar operation involving L_2 to S_3 dorsal roots was done in the case of the hind limb in 12 animals.

At various periods of time following the operation the segmental, intersegmental, and suprasegmental reflexes were studied by direct visualization and by means of moving picture and other photographic recordings in both the deafferented and intact extremities. Natural stimuli were always employed and the precise manner in which the different postural reflexes were elicited will be referred to in conjunction with the individual observations.

In order to eliminate voluntary movements which obscured the postural reflexes the animals were subjected, before the operation, to a period of training during which they became used to the manipulations employed. At the end of the period of repeated observations, which usually lasted four to six months, decerebration by the transection method (20) was performed and sacrifice experiments carried out. Also various exclusion operations were done in an attempt to analyze the suprasegmental reflexes: bilateral aseptic labyrinthectomy via the middle ear and an elimination of the neck reflexes by means of a plaster cast placed around the neck of the animal (13).

Results

The observations have been grouped into two parts. The first deals with the postural reflexes, while the second describes the disturbances of muscle tone in the deafferented extremities. All observations, unless otherwise stated, were made on chronic cats in which either one fore limb or one hind limb was permanently deafferented.

Postural Reflexes (*Static Reactions*)

1. Segmental Reflexes

A. *Positive supporting reaction* (*local static reaction*).—It was impossible to demonstrate this reflex adaption in the intact extremities of the chronic animal. Following decerebration, however, the positive supporting reaction could be readily elicited in the intact limbs. The reaction, in accordance with the observations of Magnus (13), consisted of an increase in extensor rigidity limited to the extremity to which a cutaneous stimulus was applied. Since the positive supporting reaction was dependent on the integrity of the posterior nerve roots, this reflex was permanently abolished in the deafferented limb.

Other reflexes, not essentially postural in nature but dependent like the positive supporting reaction on an intrinsic reflex arc, were also abolished in the deafferented extremity. Thus, the knee jerk elicited in the intact limb by a phasic stretch of the quadriceps was absent in the deafferented limb.

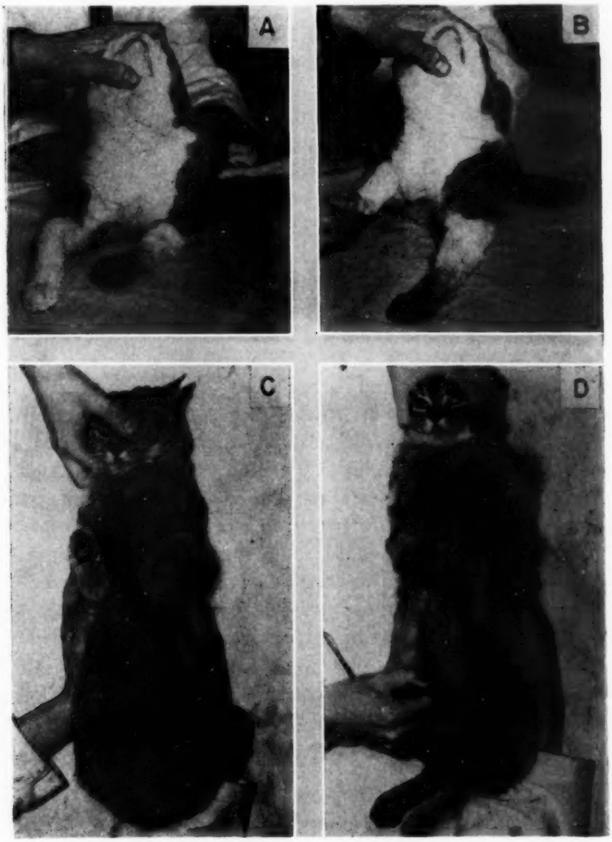

FIG. 1. Crossed extensor reflex in the chronically deafferented extremities.

A and *B*. Left fore limb deafferented 138 days (cat in prone position in horizontal plane).

A. Shows reduced extensor tone in deafferented limb when head is extended.

B. Shows on this background a crossed extensor reflex elicited by pinching the right fore paw.

C and *D*. Left hind limb deafferented 373 days (cat in supine position in horizontal plane).

C. Shows reduced extensor tone in deafferented limb when head is flexed.

D. Shows crossed extensor reflex elicited by pinching of the right hind paw.

In addition to this, disturbances of cutaneous sensation were shown by the complete loss of the flexor withdrawal in response to a nocuous stimulus applied to the deafferented extremity.

B. *Crossed extensor reflex* (*segmental static reaction*).—The crossed extensor reflex as a rule was not prominent in the extremities of intact animals and was absent immediately after section of the posterior nerve roots. On the other hand, 24–48 hr. after deafferentation the crossed extensor reflex could be readily demonstrated in both the deafferented fore and hind limbs by pinching the footpad of the contralateral leg (Fig. 1). Not only was crossed extension produced by means of cutaneous stimulation but also proprioceptive impulses from the intact limb, as evoked by passive flexion of that limb, were capable of producing an extensor response of the deafferented extremity (Philippson's Reflex (17)). Once established, the crossed extensor reflex was characterized in the deafferented limb by a brisk and forceful extension which was maintained as long as the adequate stimulus was applied to the contralateral extremity. Later in the postoperative period this reflex could be elicited by the slightest stimulation of the intact limb, being particularly exaggerated in the hind limb.

2. *Intersegmental Reflexes*

Passively induced movements or active flexion of either of the intact hind limbs produced either extension or flexion of the deafferented fore limb. These varied responses of the deafferented limbs to intersegmental stimulation were seen up to four months following deafferentation. Besides the varied postural intersegmental responses a scratch reflex was consistently produced in the deafferented hind limb. It appeared within one week following section of the posterior nerve roots and could be elicited in the deafferented hind limb but not in the intact limb of the chronic animals. After decerebration the reflex was present on both sides but was exaggerated in the deafferented extremity. In the decerebrate animal, an extensor or flexor response of the deafferented hind limb also was observed following passively induced movements of either the ipsilateral or the contralateral fore limb or when painful stimuli were applied to the footpads of these extremities.

3. *Suprasegmental Reflexes*

A. ***Tonic labyrinthine and neck reflexes acting upon the limbs—stato-tonic or attitudinal reflexes of Magnus and de Kleijn*** (*general static reactions*).—The

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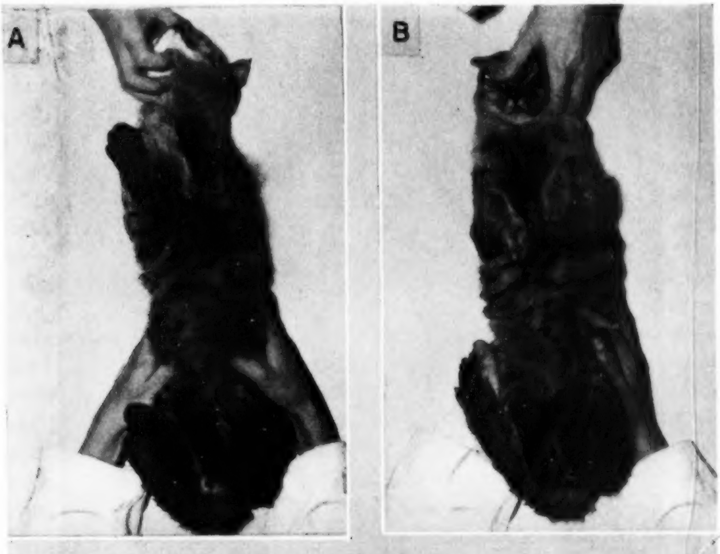
FIG. 2.—Tonic labyrinthine and neck reflexes.—Responses of fore limbs in intact cat to stato-tonic impulses in supine position.

A. With labial cleft 45° above horizontal plane—"maximal extensor position"—there is an increased extensor tone in both fore limbs.
B. Flexion of head which approaches "minimal extensor position" causes reduction of tone in the fore legs.
C. Rotation of the head to the left causes extension of the left ("jaw") limb—increased extensor tone, and flexion of the right ("skull") limb—decreased extensor tone.
D. Rotation of the head to the right reverses the position of the fore limbs and the distribution of tone in them.

Note: the absence of responses in the hind limbs with the animal in the horizontal plane (compare this with Fig. 4 *A* and *B*).

responses observed in the intact extremities to proprioceptive nerve impulses arising in the labyrinths and neck muscles were similar to those described by Magnus (13, 14). On the other hand, in the deafferented fore or hind limb these reflexes differed markedly from the ones seen in the intact limbs (Figs. 2, 3, and 4).



Immediately following unilateral section of the posterior nerve roots the tonic labyrinthine and neck reflexes were absent, not only in the deafferented limb, but also in the contralateral intact extremity. Within two weeks after section of the posterior nerve roots these reflex responses could be elicited in the intact limbs but not in the deafferented ones. One to two months after the operation the tonic neck and labyrinthine reflexes were once more present

in the deafferented fore limb, while two to three months elapsed before the same reflexes could be elicited in the deafferented hind limb. When they reappeared in the deafferented limb of the chronic animal the tonic labyrinthine and neck reflexes showed a complete reversal from the normal response. Thus, if the animal was placed in the supine position with the labial cleft inclined 45 degrees above the horizontal plane, "maximal attitudinal extensor position" of Magnus (13, 14), the intact fore limb was extended while the deafferented one became semiflexed or completely flexed (Fig. 3 *A*). Ventroflexion of the head in this position resulted in an extension of the deafferented fore limb and flexion of the intact one (Fig. 3 *B*).

Similarly, when the animal was placed in the prone position with the labial cleft inclined 45 degrees below the horizontal plane, "minimal attitudinal position" of Magnus (13, 14), the deafferented fore limb was rigidly extended while the intact limb was flexed. Intermediate degrees of rotation in the vertical plane between the "minimal" and "maximal attitudinal positions" diminished the differences in posture between the deafferented and the intact fore limbs; when the animal was midway between the two positions (i.e. rotated 90 degrees from either the "minimal" or "maximal attitudinal position") the posture was identical in both fore limbs. Rotation of the head in the longitudinal axis also resulted in different responses of the intact and the deafferented extremities. Thus, when in the supine position the head was rotated to the left this resulted in the usual flexion of the intact right or "vertex" limb and in a simultaneous flexion of the deafferented left or "jaw" limb, both extremities becoming relaxed (Fig. 3 *C*). On the other hand, rotation of the head to the right resulted in a simultaneous extension of both the intact right fore limb and of the left deafferented one (Fig. 3 *D*).

The reversal of the tonic labyrinthine and neck reflexes in the deafferented hind limb was best demonstrated by holding the animal by the scruff of the neck in the vertical plane. Ventroflexion of the head with the animal in this position resulted in the usual extension of the intact hind limb and flexion of the deafferented one, while dorsiflexion of the head resulted in flexion of the intact limb and extension of the deafferented limb (Fig. 4 *B* and *C*).

FIG. 3. Tonic labyrinthine and neck reflexes.—Responses of the deafferented fore limb to stato-tonic impulses in supine positions (left C_2–T_3 dorsal roots severed 138 days previously).

A. With labial cleft 45° above horizontal plane intact (right) fore limb is in "maximal extensor position" but the deafferented (left) fore limb is lying along the side of the body.

B. Flexion of the head causes a reduction in extensor tone of the intact fore limb coincidentally with a maximal extension of the deafferented extremity.

C. Rotation of the head to the left causes flexion of the right (skull) fore limb and a simultaneous flexion of the deafferented (jaw) limb instead of the usual extension of the latter which is shown in Fig. 2. The extensor tone in both fore limbs is greatly reduced in this position.

D. Rotation of the head to the right reverses the position of the fore limbs and the distribution of tone in them. This results in an extension of both fore limbs.

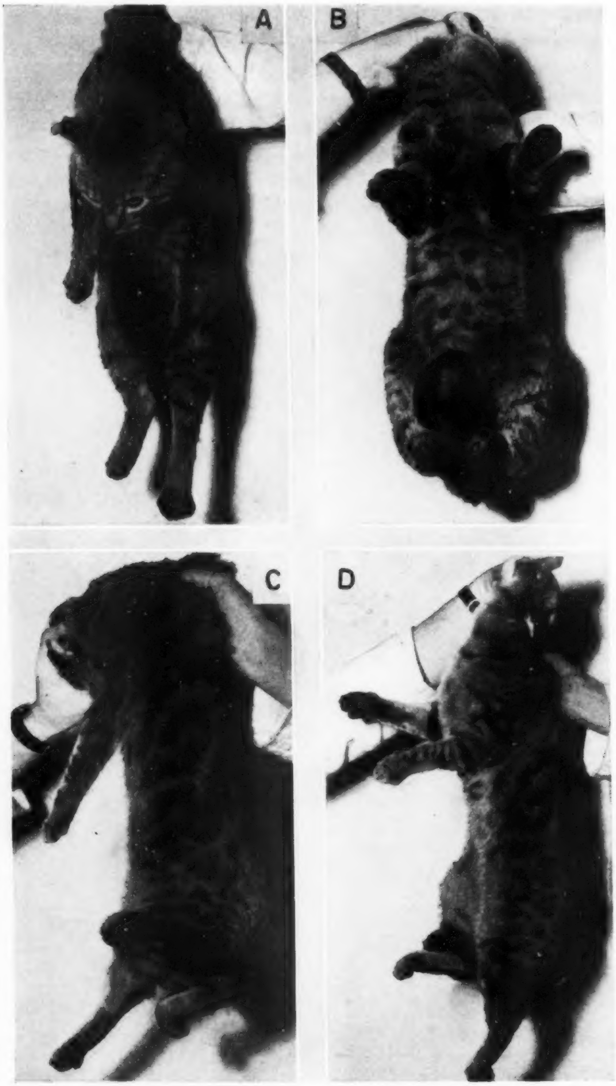


FIG. 4. Tonic labyrinthine and neck reflexes.—Responses of the intact and deafferented hind limbs to stato-tonic impulses in the vertical plane.

A. In an intact cat flexion of the head results in extension of both hind limbs and in an increased extensor tone.
B. Extension of the head leads to a flexion of the hind limbs and to a reduction of tone.
C and *D*. Show reversal of these reflexes in the left hind limb 373 days after aseptic section of the L_2–S_2 dorsal roots.

Bilateral labyrinthectomy and immobilization of the head on the trunk by means of a plaster cast abolished these reflexes both in the fore and hind limb similar to those in the normal extremity (13).

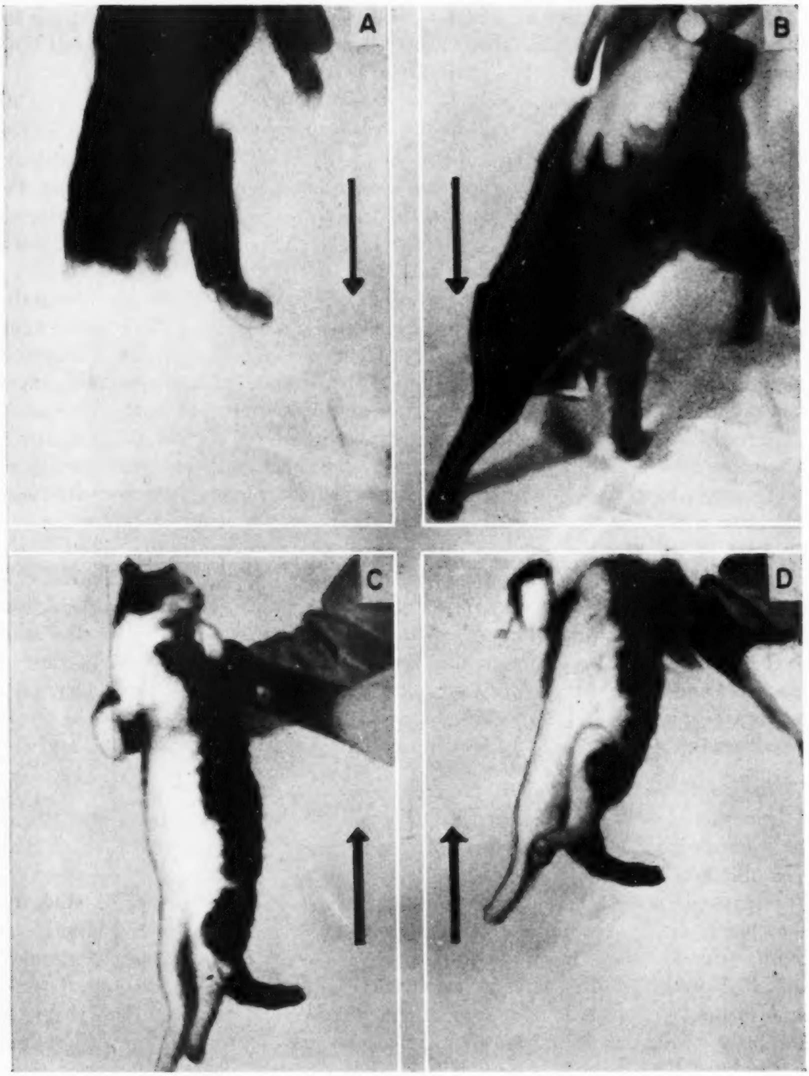
B. *Reflex responses to acceleration—Stato-kinetic or positional reflexes.*—One of the best known responses to linear acceleration was demonstrated in the intact animal by Magnus (13) and by Bard (1). If the animal was suddenly lowered to the ground, the fore limbs became extended, while raising the animal away from the ground caused the fore limbs to become flexed. Similar responses, but not as marked, occurred in the hind limbs when the animals were lowered and raised with the head upright.

Immediately following deafferentation of one fore limb or one hind limb the linear acceleratory reflexes could not be elicited either in the denervated or in the intact extremity. Two to four days after the operation the linear acceleratory reflexes returned in the intact limb. In the deafferented extremity these reflexes returned one to four weeks after the operation and once established they could be readily elicited throughout the remaining period of observation. The slightest displacement of the animal caused the deafferented extremity to be briskly and forcibly moved even when the displacement was insufficient to cause a response in the intact contralateral limb (Figs. 5 and 6). No reversal of the reflexes induced by linear acceleration was ever noted, their exaggeration apparently being the only characteristic feature after deafferentation. As in the case of the reversal of tonic labyrinthine and neck reflexes, after deafferentation the linear acceleratory reflexes reappeared earlier and were more readily elicited in the fore limb than in the hind limb. Fixation of the head and neck in a plaster cast had no marked effect on the acceleratory reflexes (Fig. 6 *A* and *B*), while bilateral labyrinthectomy abolished these responses both in the intact and in the deafferented extremity (Fig. 6 *C* and *D*).

Disturbances of Muscle Tone in the Deafferented Limb

Immediately after section of the posterior nerve roots the limb was atonic as well as showing an absence of postural reflexes; however, with the reestablishment of these reflexes tone reappeared in the deafferented extremity. Furthermore, the alterations of tone in that limb seemed to be directly associated with these reflexes and therefore could be attributed largely to them. For instance, in the postoperative period the crossed extensor reflex and Philippson's reflex (17) returned earlier in the deafferented hind limb than in the fore limb and they were more readily elicited in that limb (Fig. 1). Similarly, variations in muscle tone were noted sooner and were much greater in the hind limb than in the fore limb in the corresponding period of time following deafferentation (one to seven days).

On the other hand, the linear acceleratory reflexes were more conspicuous in the fore limb than in the hind limb and could be demonstrated in the fore limb within one week after section of the posterior nerve roots, a somewhat longer period of time after the operation (three to four weeks) being required before they were observed in the hind limb. These reflexes also influenced the tone in the deafferented limb: for instance, if the cat was rapidly raised away



from the ground, extensor tone was decreased in the extremity, while the reverse occurred when the animal was lowered toward the ground (Figs. 5 and 6). These modifications of tone in the deafferented limb in response to linear acceleration were transient in nature and were evident only when the adequate stimulus was in operation. In this they were similar to the tonic adjustments mediated by the crossed extensor reflex.

The deficiency of the crossed extensor reflex in the fore limb seemed to be compensated for by the overactivity of the linear acceleratory reflexes; this was particularly evident in the early stages of recovery from the operation. In this manner a considerable degree of muscle tone, transient in nature, was evident in both the deafferented fore and hind limbs already during the first month after section of the posterior nerve roots.

A considerably longer period of time after section of the posterior nerve roots (one to four months) was required before the influence of the tonic neck and labyrinthine reflexes on muscle tone in the deafferented limb became evident. As stated before, in the supine or "maximal attitudinal position" the deafferented fore limb was hypotonic and assumed a semiflexed or flexed position on the chest while the intact fore limb was rigidly extended (Fig. 3 *A*). With the cat in this position it was possible to alter the hypotonicity of the deafferented limb by pinching the footpad of the intact fore limb. Thus in the presence of crossed segmental stimulation the deafferented fore limb became rigidly extended while tone in the intact fore limb was considerably reduced. When the cat was placed in the prone or "minimal attitudinal position", which corresponded to the standing attitude, muscle tone in the deafferented fore limb was maximal and the limb actively resisted passive flexion while the intact fore limb was hypotonic.

Tonic labyrinthine and neck reflexes in the deafferented hind limb could not be readily demonstrated in the maximal and minimal positions and therefore as a rule a complete reversal of tone was not elicited in these positions. However, a reversal of muscle tone caused by these postural reflexes was observed in the hind limb when the animal was held in the upright position by the scruff of the neck (Fig. 4 *B* and *C*).

Discussion

The present findings as regards the crossed extensor reflex in the deafferented limbs of chronic animals are in agreement with those of Ranson (19) and Sprong (27) who described an initial depression of the deafferented neurones to crossed stimulation which was followed in 24 to 48 hr. after section of the

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FIG. 5. Positional or stato-kinetic reflexes.—Linear acceleratory reflexes in deafferented fore and hind limbs elicited in vertical plane.

A and *B*. Cat held by pelvis and lowered to the ground (Left C_2–T_2 dorsal roots severed two months previously).
Note: semiflexed position of both fore limbs at the beginning of the descent (*A*), and the exaggerated maximal extension of the deafferented fore limb when the animal reaches the ground as compared with a semiflexed position of the intact fore limb (*B*).

C and *D*. Cat held by upper part of the body and raised away from the ground (Left L_2–S_3 dorsal roots severed four months before the test).
Note: the almost symmetrical extension of both hind limbs at the beginning of the ascent (*C*), and the flexion of the deafferented (left) hind limb which becomes pronounced at the height of the ascent while the right limb remains extended (*D*).

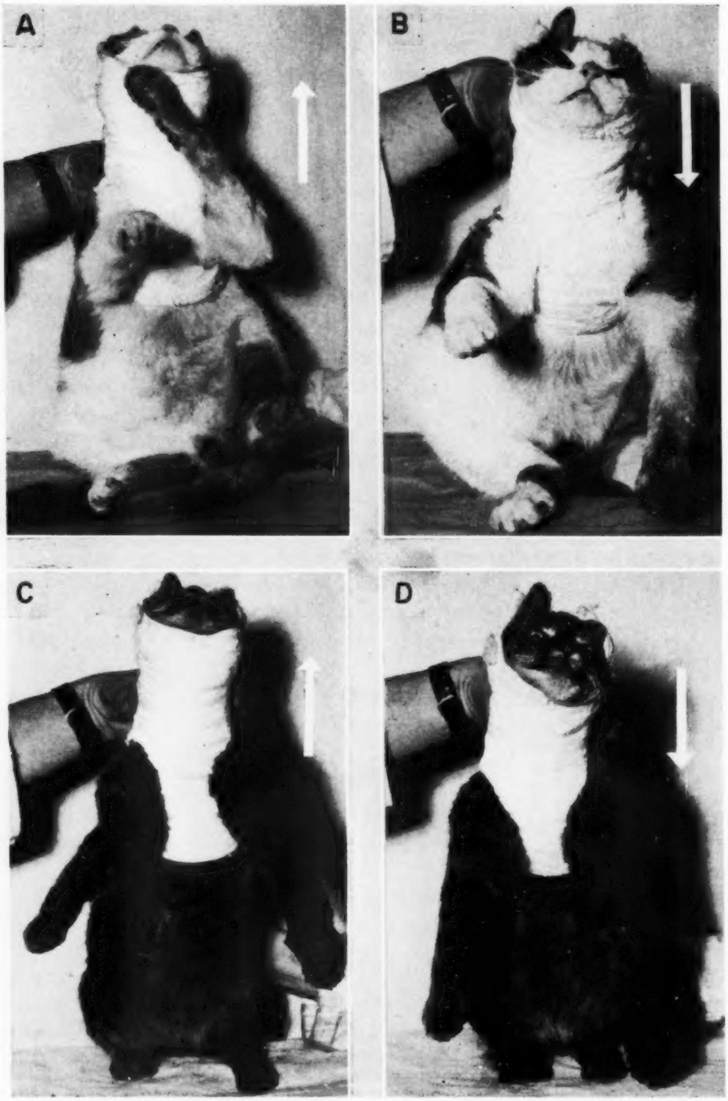

FIG. 6. Analysis of the linear acceleratory reflexes in the deafferented fore limb.

A and *B*. Show exaggerated flexion and extension of the left fore limb (deafferentation of four months' standing) as compared to the intact right limb on raising and lowering of the animal respectively. The plaster cast excludes any movement of the head relative to the body, eliminating the "neck" reflexes.

C and *D*. Show absence of these responses under similar conditions after a bilateral labyrinthectomy. (Deafferentation of the left fore limb of three months' standing. Bilateral labyrinthectomy of three weeks' standing.)

posterior nerve roots by an exaggerated crossed extension of the hind limb. On the other hand, Sherrington (22) and Fulton and Liddell (7) while studying the immediate effects of deafferentation on this reflex failed to observe any initial depression and indeed found that the reflex was more readily elicited than in the intact vastocrureus muscle. However, in the experiments of Sherrington (22) and Fulton and Liddell (7) the posterior nerve roots from the vastocrureus alone were sectioned while in our animals and in those of Ranson (19) and Sprong (27) the hind limb was completely deafferented. Thus it seems that extensive deafferentation affects this reflex in a different way from that seen on section of isolated sensory nerve roots. The finding of Luco and Eyzaguirre (12) that immediately after section of one posterior nerve root there is no depression of excitability of spinal neurones to chemical stimulating agents, while complete deafferentation of a limb was shown by Drake and Stavraky (6) to result in a profound depression of these neurones, supports such a supposition.

The exaggerated response of the acutely deafferented vastocrureus muscle was attributed by Sherrington (22) to the resulting hypotonia, while Fulton and Liddell (7) ascribed it to a lack of autogenous inhibition. Neither of these explanations could account for the failure of local anesthesia of the muscles to exaggerate the response, or for the time lag between complete deafferentation of an extremity and the appearance of hyperreflexia, as well as for other characteristics of the crossed extensor reflex seen in the chronically deafferented limb. This was pointed out by Bremer (2) and by Sprong (27) who attributed the exaggerated response and lowered threshold of the deafferented spinal neurones to an augmentation of their excitability. Similarly, Moldaver (16) described a prolonged after-discharge in the quadriceps muscle after deafferentation and concluded that such a persistence of activity must be related to an increased excitability of the deafferented spinal neurones to impulses reaching them from the opposite side of the spinal cord, while Drake and Stavraky (6), Luco and Eyzaguirre (12), and Teasdall and Stavraky (28) demonstrated a hyperreactivity of chronically deafferented spinal neurones to chemical stimulating agents and to physiological nerve impulses traversing intact synaptic pathways. The alteration of neuronal excitability brought about by deafferentation was attributed by these investigators to a supersensitivity brought about by denervation in accordance with the Bernard–Cannon Law (3). It seems most likely that the exaggerated responses of the chronically deafferented spinal neurones to impulses reaching them from the opposite side of the spinal cord in the case of the crossed extensor reflex may also be due to such a sensitization.

In regard to the intersegmental reflexes Sprong (27) noted that extension of the contralateral fore limb was usually associated with strong extension of the deafferented hind limb, while flexion of the contralateral fore limb or extension of the fore limb on the same side as the deafferented hind limb led to less definite responses. In a similar study Pollock and Davis (18) stated that the

intersegmental reflexes were hyperactive in the deafferented hind limb. In the present investigation, in addition to this finding, the scratch reflex was found to be greatly exaggerated in the deafferented hind limb of chronic animals. Thus it may be concluded that the spinal neurones were sensitized by the preceding deafferentation to reflex impulses reaching them from higher levels of the spinal cord as well as to segmental ones and to impulses reaching them via the pyramidal system (28).

The linear acceleratory reflexes were also exaggerated and could be elicited more readily in the deafferented limb than in the intact extremity of the chronic animal. Hyperreactivity of the deafferented extremity to linear acceleration was described previously in pigeons (29), and in cats (2, 19, 27, 18). Not only were the linear acceleratory reflexes found to be hyperactive in the deafferented limb, but also a decreased threshold of excitation was demonstrated in the present investigation. These findings may be attributed to the sensitization of the deafferented spinal neurones to vestibulospinal impulses. Such a conclusion is supported by the fact that an exaggeration of responses mediated by the vestibulospinal paths in cats with cerebellar lesions was demonstrated by Cook and Stavraky (5).

The reversal of the tonic labyrinthine and neck reflexes noted during the present investigation in the chronically deafferented fore and hind limbs is of interest. Sherrington and Sowton (25) recording from the limbs responses of the isolated vastocrureus in the decerebrate cat found that strong electrical stimulation of an ipsilateral afferent nerve caused flexion, while weak stimulation of the same nerve resulted in extension of the limb. Analogous reversals were recorded by these authors also in the case of the flexors of the knee (26). Sherrington and Sowton suggested that this type of reflex reversal may be due to the presence of inhibitory and excitatory fibers of different thresholds in the afferent nerves. The fact that reflex reversal may be brought about by different patterns of stimulation was substantiated by Wyss (30) working on the respiratory center but was attributed to a central mechanism. Hughes, Stavraky, and Teasdall (8) showed that sensitization of spinal neurones by preceding semidecerebration greatly facilitated the occurrence of spinal reflex reversals, thus further bearing out the central nature of the phenomenon. They suggested that reflex reversal was related to an increment of central excitation. A similar mechanism may have operated in the reversal of the tonic labyrinthine and neck reflexes seen in the deafferented limbs of chronic animals in the present study. When the cat was placed in the supine position the labyrinthine proprioceptors were maximally stimulated. The resultant excitation of the intact spinal neurones by impinging vestibulospinal impulses produced extension of the corresponding limb, while a similar excitation of neurones sensitized by a preceding section of the dorsal roots was excessive and led to an inhibition of the neuronal pool—the extensor tone of the deafferented fore limb becoming reduced. If the animal was placed in a prone position stimulation of the labyrinthine proprioceptors was minimal, the

vestibulospinal impulses thus induced were insufficient to cause a discharge of the spinal neurones innervating the extensor muscles of the intact fore limb and accordingly that limb remained in a flexed position. On the other hand, owing to the sensitization of the deafferented neurones on the opposite side of the cord these neurones were excited by the weak vestibulospinal volley and accordingly the deafferented fore limb extended. Therefore, the reversal of the tonic labyrinthine and neck reflexes in a chronically deafferented limb may be attributed to sensitization of the deafferented spinal neurones to vestibulospinal impulses. It is significant in this respect that in an investigation of children with amaurotic familial idiocy de Kleijn (9) described a disturbance of these reflexes which was possibly related to that seen in the present study but he was unable to account for it adequately at that time.

On the other hand, Sherrington (23), Ranson (19), Sprong (27), and Pollock and Davis (18) noted an exaggeration of the tonic labyrinthine and neck reflexes in the deafferented limb but failed to observe the reversal of these reflexes. This may be due to the fact that sufficient time was not allowed after section of the posterior nerve roots for the reversal to develop in the deafferented extremity. Also these reflexes were studied by the above investigators in the deafferented hind limb in which the reversal was much more difficult to elicit and could be more clearly demonstrated when the animals were placed in the vertical plane. This relative difficulty in eliciting the tonic neck and labyrinthine reflexes in the deafferented hind limb was undoubtedly related to the fact that different postural reflexes oppose each other in the lower extremity but act synergistically in the fore limb (13). The maximal facilitation of reflexes in the lower extremity which occurred in the vertical plane is of interest. It is easy to see that such a selective facilitation of reflexes would be of an advantage to the animal when landing on its hind limbs in the case of a fall. The mechanism of this facilitation is probably labyrinthine in nature, however it has not been analyzed in any detail at this time. Finally, the fact should be considered that only the tonic labyrinthine and neck reflexes showed complete reversal while the crossed extensor, linear acceleratory, and the scratch reflex in the deafferented extremities were greatly exaggerated but not reversed. The inability to produce a sufficient intensity of afferent stimulation under given experimental conditions probably accounts for this finding.

As a result of the alterations in the postural reflexes muscle tone in the deafferented limb differed greatly from the tonic adjustments which occurred in the intact extremity. Owing to the interruption of the intrinsic proprioceptive reflex arc the acutely deafferented limb was at first hypotonic (4, 2, 27). However, this hypotonicity of the deafferented extremity was not a permanent feature and variations in muscle tone were observed coincident with the return of the extrinsic postural reflexes. Accordingly, it may be concluded that in the absence of the intrinsic proprioceptors the supersensitivity of the deafferented spinal neurones to segmental, intersegmental, and to suprasegmental postural reflexes compensated in part for the deficiency of tone in the

chronically deafferented limb. In spite of this, however, the limb deprived of its posterior nerve roots was never capable of attaining the constancy of tone which is characteristic of the intact extremity.

The tonic labyrinthine and neck reflexes were capable of maintaining a certain degree of tone in the deafferented extremity as long as the head was kept in a constant position both in relation to space and to the trunk, but in view of their reversal, this tone differed in distribution from the one present in the intact limb and was influenced quite differently from the normal tone by the crossed extensor and linear acceleratory reflexes. It is interesting to note that the tonic adjustments mediated by the labyrinthine and neck reflexes and by the linear acceleratory reflexes returned much sooner in the deafferented fore limb and were more readily elicited in that extremity than in the hind limb. On the other hand, the crossed extensor reflex was more prominent in the deafferented hind limb. This suggests that tone in the fore limbs was much more dependent on reflex influences from higher levels of the central nervous system while tonic adjustments of the hind limbs were mediated essentially at the spinal level.

The disturbances of muscle tone which became apparent in the chronically deafferented limb were closely associated with abnormal motor responses. These amounted to a gross incoordination of movements in the deafferented extremity which resembled closely clinical sensory ataxia. It is commonly asserted that the interruption of the intrinsic proprioceptive reflex arc is the cause of this disturbance. However, as pointed out by Liljestrand and Magnus (11) and by Bremer (2) novocainization of the muscles fails to induce ataxia. Similarly, acute transection of the posterior nerve roots does not result immediately in typical ataxia which requires some time for its development. From the findings of Liljestrand and Magnus (11) and Bremer (2), it became apparent that the onset of ataxia is related to the appearance of exaggerated reflex responses rather than to the interruption of the proprioceptive reflex arc. This was further brought out by the studies of Moldaver (16) who like Bremer suggested that sensory ataxia develops as the result of hypersensitivity of spinal neurones to reflex stimulation. This view is in agreement with the findings of Drake and Stavraky (6) and Teasdall and Stavraky (28) who showed that chronically deafferented spinal neurones respond in an exaggerated manner to chemical stimulation and to impulses reaching them via the corticospinal tract.

From the present study it becomes apparent that a profound disturbance of attitudinal postural reflexes develops in deafferented extremities. This is another important factor which undoubtedly contributes to the development of hypermetria in human posterior nerve root involvement. Thus, sensory ataxia must be regarded as the result of a composite series of changes in which alterations in tone, exaggeration of the responses of the spinal neurones to voluntary impulses reaching them via the corticospinal tracts, and the disturbance of reflex postural adjustments on the background of which these impulses exert their effect all play their part.

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EFFECT OF LIGATION OF THE COMMON DUCT ON THE RADIOIODINE UPTAKE BY THE THYROID OF RATS[1]

By K. Kowalewski, A. W. Mooney, and H. A. Hyde

Abstract

The enterohepatic circulation of radioiodine in rats was interrupted by the ligation of the common bile duct. Significant reduction in the radioactivity of the thyroids was noted in rats following ligation of the common duct and administration of radioiodine per os. In all animals on which operations were performed radioactivity of the liver was increased. The rate of excretion of radioactive bile in rats with cannulated bile ducts was also investigated. The importance of hepatobiliary factors in the process of utilization of tracer dose of radioiodine is briefly discussed. The possibility of alterations in radioiodine uptake in man with disturbed liver function is emphasized.

Introduction

It has been demonstrated previously that the liver and gastrointestinal tract concentrate radioactive material following the injection of radiothyroxine (1, 2, 3). The radioactive material is transferred from the liver by the bile to the gastrointestinal tract where partial reabsorption takes place.

In view of the fact that the bile is the agent of transport of radiothyroxine excreted by the liver, the ligation of the bile duct blocking this transport must influence the distribution, retention, and loss of the radioactivity of the experimental animal. It has been shown that ligation of the common bile duct prolonged the period of depletion of radioactivity from the tissues of animals so treated.

A study of the intracellular localization of labelled thyroxine indicated that the hormone is actively concentrated by the liver (6). It has been further demonstrated that triiodothyronine may be transferred by the bile to the gastrointestinal tract (7).

It is apparent from the foregoing investigations that the enterohepatic circulation of thyroid hormones is a reality and plays an important role in their metabolism. Subsequent studies may explain the role of liver cells in the formation, storage, and excretion of the thyroid hormones. It is possible that not only the presence or quantity of the bile determines the extent of enterohepatic circulation of injected or ingested iodine, but also the state of liver cells and quality of bile influences the thyroid excretion of hormones. It is possible that the difficulties encountered in the interpretation of some results of diagnostic radioiodine uptake are due to an abnormal enterohepatic circulation of the ingested tracer dose.

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Contribution from McEachern Cancer Research Laboratory and the Department of Surgery, University of Alberta, Edmonton Alberta.

The purpose of the present study was to determine whether the experimental alteration of enterohepatic circulation may influence the uptake of radioiodine by the thyroids of rats. Obstructive jaundice was produced in the animals by ligation of the common duct. The radioactivity of thyroids, livers, and blood was determined 24 hr. after the administration of radioactive material per os. Similar studies were done in the normal controls and in rats on which sham operations were performed.

Experimental

Male rats of the Wistar strain, varying in weight from 160 to 280 gm. were used. The rats in this laboratory are fed ad libitum with commercial Purina Dog Chow. All animals received carrier-free radioiodine (I^{131}) per os, by ball tipped metal cannula attached to a syringe. Two milliliters of aqueous solution containing 5 or 10 microcuries (μc.) were given 24 hr. before the sacrifice of the animals. A group of 32 rats had the common duct ligated four to six days before the administration of radioiodine (I^{131}). We used animals in a relatively recovered state as far as operative shock was concerned and considered this important. For their study of radiothyroxine (1, 2) other workers utilized rats on which operation had recently been performed.

To exclude possible cause of error a group of eight rats had a laparotomy performed five days before the experiment without ligation of the common duct. The third group of 28 normal rats served as another control.

In several other animals, the bile duct was cannulated with polyethylene tubing, and after intragastric instillation of radioiodine the bile was collected directly into aluminum planchets for the counting. This supplementary experiment was carried out to measure quantitatively the biliary secretion of radioiodine (I^{131}) in our experimental conditions. In this group, only in a few animals, investigation was performed four hours after cannulation.

All operations were done under nembutal, which apparently does not influence the uptake of radioiodine in rats (8). Twenty-four hours after the administration of radioiodine (I^{131}) the blood was aspirated from the inferior vena cava into heparinized syringes and plasma separated by centrifugation. The livers were perfused in situ by normal saline and dissected. The thyroids were dissected and both organs immediately weighed. The total thyroid from each rat was squashed on a circle of filter paper placed on an aluminum planchet under a single covering layer of scotch tape (4). Thyroid radioactivity determinations were carried with a thin mica window Tracerlab G–M. tube. The livers were homogenized in 20–30 ml. of 10% sodium hydroxide and 10 ml. of warm suspension was taken for counting. One milliliter of plasma was made up to 10 ml. with distilled water and counted in a liquid counter by the same method as the liver suspension. Tracerlab Superscaler S.C. 18, was used in this experiment. The results are presented in counts per minute (c.p.m.) and the percentage of administered dose.

TABLE I

RADIOACTIVITY OF THE THYROID GLANDS 24 HR. AFTER INTRAGASTRIC INSTILLATION OF RADIOIODINE

Condition	No. rats	Av. weight, gm.	Dose given, μc.	C.p.m./mgm. thyroid Av. S.D.	C.p.m./mgm. thyroid Range	Total thyroid mean uptake, % dose
Normal	13	204	10	2046 ± 354	(1856–2900)	5.25
	15	188	5	890 ± 112	(690–1240)	4.70
Common duct ligated	18	216	10	977 ± 321	(890–1820)	2.40
	14	170	5	293 ± 125	(170– 515)	1.96
Sham operation	8	180	5	758 ± 153	(540–1050)	3.94

Results

Table I gives the resulting radioactivity in the thyroids. It is noted that the total radioactivity of thyroids in rats with ligated bile ducts was below the level found in normal controls. The blocking of enterohepatic circulation of radioactive material seems to be the cause of this diminished uptake in rats with ligated bile ducts. The influence of surgical shock was apparently negligible, as the animals recovered sufficiently before the ingestion of radioiodine. The results obtained with rats that had sham operations approach those found in normal controls. Tables II and III summarize the results of counting total radioactivity in the livers and plasma of rats, respectively. It must be remembered that the sensitivity of the liquid counter used in this study was different from the sensitivity of the instrument used for the counting of radioactivity in thyroids. That is why the values of the counts per minute (c.p.m.) presented in Table I are not comparable with those given in Tables II and III. When the mean uptake of radioiodine was calculated in percentage of dose this difference of counters was corrected.

TABLE II

RADIOACTIVITY OF THE LIVER OF RATS 24 HR. AFTER INTRAGASTRIC INSTILLATION OF RADIOIODINE

Condition	No. rats	Dose given, μc.	C.p.m./gm. liver Av. S.D.	C.p.m./gm. liver Range	Total liver mean uptake, % dose
Normal	13	10	123 ± 32	(83–187)	2.4
	15	5	68 ± 14	(51– 95)	2.1
Common duct ligated	18	10	407 ± 84	(261–536)	8.7
	14	5	217 ± 38	(151–292)	8.0
Sham operation	8	5	71 ± 16	(49– 90)	3.1

TABLE III

PLASMA RADIOACTIVITY 24 HR. AFTER INTRAGASTRIC INSTILLATION OF RADIOIODINE

Condition	No. rats	Dose given, μc.	C.p.m./ml. plasma Av. S.D.	C.p.m./ml. plasma Range	Plasma mean concentration, % dose/ml.
Normal	13	10	219 ± 21	(190–259)	0.44
	15	5	139 ± 30	(81–176)	0.55
Common duct ligated	18	10	218 ± 23	(185–256)	0.43
	14	5	149 ± 31	(97–196)	0.59
Sham operation	8	5	138 ± 25	(79–168)	0.55

It is noteworthy that in all animals with the common duct ligated, the concentration of radioactivity in the livers was higher than in the control group and in the group that had sham operations. This was apparently due to the retention of radioactive bile. No significant difference in plasma radioactivity was noted between the groups studied.

Table IV shows the rate of secretion of radioactive bile in three rats. In several rats the common duct was cannulated but only in three animals was the collection of bile for four hours carried out successfully without technical difficulties. Cumulative four-hour biliary excretion was from 7.7 to 8.9% of activity of administered dose. Significant radioactivity of the bile was noted as early as five minutes after the administration of I^{131}. In two rats which did not survive the four-hour experiment the c.p.m. in the samples

TABLE IV

RADIOACTIVITY OF BILE IN RATS WITH CANNULATED COMMON DUCTS

Time of collection after ingestion of I^{131}, min.	C.p.m. in collected sample Rat 1	Rat 2	Rat 3
0– 15	420	—	—
15– 30	1498	—	—
30– 45	1780	—	—
45– 60	1956	—	—
0– 60	—	4230	4400
60– 80	3800	4816	3100
90–120	4400	4660	4720
120–150	3760	3580	3320
150–180	3100	3118	2440
180–210	3140	1248	1750
210–240	1140	1200	1870
% of activity excreted in 4 hr.	8.9	8.0	7.7

collected in the first five minutes after the instillation of I^{131} was respectively 238 and 310. Those results confirm the previous findings in animals (5) and man with biliary fistula (1), and help in the appreciation of the importance of biliary factors in the process of enterohepatic circulation.

Conclusions

In this study only total radioactivity was determined and the chemical state of measured I^{131} was not investigated. As no exogenous thyroid hormone was introduced with radioiodine, our results cannot be compared with those obtained by other observers of the enterohepatic circulation of radiothyroxine. But even without measuring the endogenous hormones bound with ingested radioiodine one may conclude that a notable alteration in uptake of I^{131} may occur in the absence of bile. It was found in this experiment that the uptake of radioiodine by the thyroids of rats may be reduced when the animals were subjected to common bile duct ligation. The abnormal retention of radioactivity in the livers of animals with ligated ducts was a constant finding independent of the administered dose. This retention was apparently due to the absence of excretion of radioactive bile. The excretion of radioactive bile was demonstrated in the animals with cannulated common ducts.

If the chemical state of measured radioiodine is influenced by the ligation of common bile duct it cannot be proved in this experiment.

It is pointed out that the dose of 5 μc. administered to these rats is very high as compared with the doses used in man for the purpose of study of radioactivity in the thyroid. It is reasonable to presume that an enterohepatic circulation of radioiodine bound or not with endogenous hormone occurs in patients given a tracer dose. Any alteration of liver function, changes in quantity or quality of excreted bile, and changes in the portal and lymphatic circulation should be seriously considered as one of the causes of abnormal uptake and distribution of radioiodine given as a diagnostic tracer dose.

The abnormal total radioactivity of thyroid after the administration of radioiodine in the presence of interrupted enterohepatic circulation was demonstrated in this experiment. These findings reflect only one aspect of dysfunction. Further studies are in progress to investigate the possible effect of liver damage and of abnormal biliary excretion on the formation and utilization of thyroid hormones.

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FURTHER STUDIES ON CARBOHYDRATE METABOLISM IN THE VITAMIN-B_6-DEPRIVED RAT[1]

By John R. Beaton

Abstract

Earlier studies in this laboratory demonstrated abnormalities in carbohydrate metabolism in the vitamin-B_6-deprived rat. The results of further studies are reported in this communication. Following three weeks of vitamin B_6 restriction, rats have significantly elevated levels of inorganic phosphorus and glutathione in blood and liver. These elevations in blood inorganic phosphorus and glutathione levels have been similarly demonstrated after only one week of vitamin B_6 deprivation. Contrary to changes in liver glycogen levels, muscle glycogen levels are not altered by vitamin B_6 deprivation in the rat. Insulin administration had a slightly more pronounced effect on blood sugar levels in vitamin-B_6-deprived than in pair-fed control rats. Alloxan administration elevated blood sugar levels of deprived rats to a slightly greater extent than the levels of controls. In accord with the earlier studies, disturbances of carbohydrate metabolism can be readily demonstrated in vitamin-B_6-deprived rats.

Introduction

Vitamin B_6 has been implicated for several years in the metabolism of amino acids (3, 17, 18) and of fat (6, 15) although a possible role in carbohydrate metabolism has been largely neglected. Recent studies in this laboratory (4) have suggested an abnormality in carbohydrate metabolism of rats deprived of vitamin B_6 and compared with pair-fed controls. This abnormality was typified by significantly lowered fasting levels of liver glycogen, blood sugar, blood pyruvic acid, and blood lactic acid following three weeks of vitamin B_6 restriction. With a deprivation of only one week duration, significantly lowered fasting levels of blood sugar and liver glycogen were demonstrated. A significant decrease in the activity of hepatic lactic acid dehydrogenase was also noted in vitamin-B_6-deprived rats. These findings have led to further studies on carbohydrate metabolism which are reported in this communication.

Methods

In all cases, young albino rats of the Wistar strain and of both sexes were housed in individual, screen-bottomed cages and provided with a 20% corn oil, 20% casein, vitamin-B_6-free basal diet (3). Drinking water was freely available. Control animals were given 50 μgm. of pyridoxine hydrochloride per rat per day in their food and were pair-fed with their comparable deprived groups to eliminate differences in analytical results consequent to differences in the amount of food consumed. Following the indicated period of experimental feeding the rats were fasted for 18 to 20 hr. and anesthetized with an

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intraperitoneal injection of 2% nembutal in 0.9% saline. Care was taken to avoid causing any increase in activity by this procedure. Blood was removed from the exposed heart with a hypodermic syringe, heparinized, and individual determinations done for glutathione (9), inorganic and acid-soluble phosphorus (8), and sugar (16). In one experiment, glycogen was determined in skeletal muscle (abdominal) by the procedure of Kahan (11). Inorganic phosphorus was determined in 3% extracts of liver in 10% trichloracetic acid (8) and glutathione was determined in liver extracts (9). A "t" test has been applied to the results to ascertain the significance of differences between group means.

Experimental

Muscle Glycogen Levels in Vitamin-B_6-deprived Rats

Thirty-eight rats were divided into four groups of nine or 10 rats each with an initial average body weight of 104 gm. Following each of 7 and 21 days of experimental feeding, one deprived and one control group were fasted, anesthetized, and about 400 to 500 mgm. of abdominal muscle extracted with 5% trichloroacetic acid with the aid of acid-washed sand and a pestle and mortar to yield a 10% extract. This extract was filtered and its glycogen content determined. The results of this study are shown in Table I.

TABLE I

MUSCLE GLYCOGEN LEVELS IN VITAMIN-B_6-DEPRIVED AND PAIR-FED CONTROL RATS

Group	Days on diet	Av. daily food intake, gm./rat	Av. body weight gain, gm.	Muscle glycogen mean ± S.D., mgm.%	t
Deprived	7	13.0	23	570 ± 70 (10)	0.38
Control	7	13.0	37	510 ± 120 (10)	
Deprived	21	13.6	54	720 ± 320 (9)	0.17
Control	21	13.6	95	630 ± 240 (9)	

( ) *Signifies the number of rats per group.*

Fasting Levels of Blood and Liver Glutathione and Phosphorus in Vitamin-B_6-deprived Rats

Thirty-six rats were divided into four groups with an initial average body weight of 98 gm. Following each of seven and 21 days of experimental feeding, one deprived and one control group were fasted, anesthetized, and glutathione, inorganic phosphorus, and acid-soluble phosphorus determined in individual blood samples. Liver glutathione and inorganic phosphorus levels were also determined in the animals sacrificed after 21 days of experimental feeding. The results of these analyses are shown in Table II.

TABLE II

BLOOD AND LIVER INORGANIC PHOSPHORUS, ACID-SOLUBLE PHOSPHORUS, AND GLUTATHIONE LEVELS IN VITAMIN-B_6-DEPRIVED AND PAIR-FED CONTROL RATS (9 RATS PER GROUP)

	Days on diet	Deprived	Control	t
Blood inorganic phosphorus, mgm.%	7 21	7.3 ± 0.5 6.7 ± 0.5	6.6 ± 0.6 5.7 ± 0.5	2.69† 4.00*
Blood acid-soluble phosphorus, mgm.%	7 21	33.3 ± 4.4 32.3 ± 2.8	27.4 ± 7.3 29.2 ± 4.2	2.07 1.73
Blood glutathione, mgm.%	7 21	29.5 ± 4.4 17.0 ± 1.5	23.3 ± 2.1 12.1 ± 2.4	3.80* 4.85*
Liver inorganic phosphorus, mgm./100 gm.	21	39.5 ± 4.6	29.0 ± 2.5	5.71*
Liver glutathione, mgm./100 gm.	21	47.6 ± 8.6	28.4 ± 5.5	5.34*
Av. daily food intake, gm./rat	7 21	14.0 13.3	14.0 13.3	
Av. body weight gain, gm.	7 21	33 49	37 92	

* *Significant at the 1% level.*
† *Significant at the 5% level.*

Insulin Administration to Vitamin-B_6-deprived Rats

As a test of carbohydrate metabolism in the vitamin-B_6-deprived rat, the effect of insulin on blood sugar levels was studied. Again comparison was made with pair-fed controls. Forty-six rats were divided into six groups of seven or eight rats each with an initial average body weight of 101 gm. Following 21 days of experimental feeding, during which time the average

TABLE III

BLOOD SUGAR LEVEL IN VITAMIN-B_6-DEPRIVED AND PAIR-FED CONTROL RATS FOLLOWING INSULIN ADMINISTRATION

Hours after insulin injection‡	Blood sugar, mgm.%		t
	Deprived Mean ± S.D.	Control Mean ± S.D.	
0	105 ± 18 (8)	120 ± 20 (8)	1.57
1.5	65 ± 11 (7)	94 ± 21 (7)	3.05*
4	84 ± 16 (8)	105 ± 16 (8)	2.63†

* *Significant at the 1% level.*
† *Significant at the 2% level.*
( ) *Signifies the number of rats per group.*
‡ *One unit zinc-insulin per kgm. body weight by subcutaneous injection.*

daily food intake of all groups was 16 gm. per rat, average body weight gains were 67 and 93 gm. for deprived and control groups respectively. All rats were fasted for 18 hr. and fasting blood sugar levels were determined on heart blood from one deprived and one control group following anesthetization. The remaining two deprived and two control groups were injected subcutaneously with zinc-insulin (Connaught Medical Research Laboratories) in 0.9% saline to provide one unit per kilogram body weight. At each of 1.5 and 4 hr. after insulin administration, one deprived and one control group were anesthetized and individual blood sugar levels determined on heart blood. The results of this investigation are set down in Table III.

Alloxan Administration to Vitamin-B_6-deprived Rats

Thirty-six rats were divided into four groups with an initial average body weight of 102 gm. Following 24 days of experimental feeding, one deprived and one pair-fed control group were given alloxan monohydrate in saline by subcutaneous injection at a level of 75 mgm. per kilogram body weight. This dosage level was found by Grunert and Phillips (10) to just fail to produce diabetes in normal rats, using a blood sugar level of 175 mgm.% as indicating diabetes. Experimental feeding was continued for a further three days. The rats were then fasted, anesthetized, and sugar determined in individual blood samples. The results are set down in Table IV.

TABLE IV

THE EFFECT OF ALLOXAN ADMINISTRATION ON BLOOD SUGAR LEVELS OF VITAMIN-B_6-DEPRIVED AND PAIR-FED CONTROL RATS (9 RATS PER GROUP)

Group	Av. daily food intake, gm./rat	Av. body weight gain, gm.	Blood sugar, mgm.% Mean ± S.D.	t
Deprived	12.5	62	73 ± 25	2.94*
Control	12.5	101	100 ± 11	
Deprived† alloxan	14.0	62	121 ± 15	0.869
Control† alloxan	14.0	119	128 ± 18	

* *Significant at the 1% level.*
† *Alloxan monohydrate 75 mgm. per kgm. body weight by subcutaneous injection.*

Results and Discussion

To facilitate discussion of results, each experiment will be treated separately. As shown in Table I no significant difference in mean muscle glycogen levels were observed between deprived and pair-fed control animals and a relatively large individual variation existed within each group. This finding is of interest in that significantly lowered liver glycogen levels were previously noted in vitamin-B_6-deprived rats after both one and three weeks of vitamin

restriction (4). The lack of an alteration in skeletal muscle glycogen level might be attributable to the fact that muscle glycogen is less labile than liver glycogen and is not readily converted to blood glucose.

The data in Table II demonstrate that following either one or three weeks of vitamin B_6 deprivation, rats have significantly elevated fasting blood levels of inorganic phosphorus and glutathione. Although an elevation in blood acid-soluble phosphorus was also found, this elevation did not attain statistical significance. Liver inorganic phosphorus and glutathione levels were similarly elevated after three weeks of vitamin restriction. Kaplan (12) has given an excellent summary of knowledge relating phosphorus to carbohydrate metabolism and has stated that an accumulation of inorganic phosphorus accelerates the rate of burning of carbohydrate and is unfavorable for glycogen synthesis. The elevated inorganic phosphorus level of blood and liver in vitamin-B_6-deprived rats supports the hypothesis of accelerated catabolism of carbohydrate. Ling and Chow (14) have recently demonstrated an inverse relationship between blood glutathione and sugar levels in vitamin-B_{12}-deficient rats. In the present studies it is of interest that vitamin-B_6-deprived rats have a significantly lowered blood sugar level and a significantly elevated blood glutathione level. A direct relationship between carbohydrate metabolism and glutathione has been demonstrated by Krimsky and Racker (13) who found that glutathione is the prosthetic group of 3-phosphoglyceraldehyde dehydrogenase. Similarly Cavallini (7) has postulated that glutathione plays a role in the coupled oxidative decarboxylation of pyruvate. The present observations on blood and liver levels of glutathione in the vitamin-B_6-deprived rat suggest that these animals are at least capable of rapidly catabolizing carbohydrate.

Following insulin administration to vitamin-B_6-deprived and pair-fed control rats (Table III) a significant depression in blood sugar level occurred in both groups (for deprived, $t=4.66$; for control, $t=2.33$). Four hours after insulin administration, blood sugar was still significantly depressed in vitamin-B_6-deprived rats ($t=2.43$) but not in controls ($t=1.67$). At zero time the mean fasting blood sugar level of deprived rats was lower than that of controls but unlike earlier studies this depression did not attain significance owing to the wide individual variation. The data following insulin administration suggest that the return of blood sugar toward the initial level is slower in deprived than in control rats possibly as a consequence of an increased catabolism or decreased formation of carbohydrate. It should be noted that convulsions were not observed in any of the insulin-treated animals.

Following alloxan administration (Table IV), diabetes, as indicated by a fasting blood sugar level of 175 mgm.% (10), was not produced in either control or deprived rats although significant elevations in mean blood sugar were noted in both control ($t=3.99$) and deprived rats ($t=4.80$). The initial fasting blood sugar level of deprived rats was significantly lower than in pair-fed controls but with the administration of alloxan this difference between groups was not present.

In agreement with earlier observations from this laboratory (4) the present findings support the hypothesis that rats deprived of vitamin B_6 catabolize carbohydrate to a greater extent than do control rats. Since a paired-feeding technique has been used throughout these studies, this effect on carbohydrate metabolism is not due to a difference in the amount of food consumed between groups. Similarly, absorption of carbohydrate by vitamin-B_6-deprived rats has been shown to be normal (6). Several of the alterations in carbohydrate metabolism occur after only one week of vitamin restriction. It should be noted that alterations in amino acid metabolism do not occur until after three weeks of vitamin B_6 restriction (1). It may well be that these metabolic abnormalities are secondary to a fundamental disturbance in energy production or utilization although thyroid activity is normal in these rats (2, 5).

Acknowledgments

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LIFE SPAN OF MICE FED A HIGH FAT DIET AT VARIOUS AGES[1]

By Ruth Silberberg and Martin Silberberg

Abstract

The effect of a high fat diet on life span depends on strain and sex of the animal tested and is specifically due to the fat content, and not to the high caloric value, of the diet. In male mice of strain C57BL, feeding of a high fat diet from the ages of 1, 6, or 12 months on through life shortened the life span. If the high fat diet was given for a limited period of time, the effect on life span depended on the age at which these mice were placed on the high fat ration.

Feeding a diet containing 30% fat to male mice of strain C57BL from the time of weaning through life significantly shortened the life span of these animals as compared to that of controls fed a stock ration containing 5% fat (6). In continuation of these investigations, it was thought of interest to record the effect on life span of a fat-enriched diet given at various ages and for different periods of time.

Material and Methods

Four hundred and forty-one male mice of the closely inbred strain C57BL raised in our laboratory were observed. One group of animals fed the stock diet of Purina Laboratory Chow through life (group I) and another group fed the high fat diet from weaning through life (group II) served as controls. Five groups of mice were given the high fat diet for limited periods of time. In two of these groups, the fat-enriched ration was fed to the mice from the age of six months (group III) and 12 months (group IV) on through life; to the remaining three groups of animals, the diet was given for a period of five months starting at the ages of one month (group V), seven months (group VI), or 12 months (group VII), respectively. Up to the beginning and on through life from the end of these five months' periods, the animals received the stock diet. Both rations were given ad libitum with water available at all times. The distribution of the animals in the various groups is shown in Table I.

The high fat ration was prepared by grinding the Laboratory Chow to a meal and adding 25% lard (Swift's Silverleaf Brand). The exact composition of this diet, which is adequate in all respects and which is well tolerated, has been given previously (5).

Since the experiments were primarily designed for the study of skeletal changes, a number of animals were sacrificed at predetermined ages. The latter mice have been disregarded in the present analysis, and only those 225 animals that died or had to be killed because of sickness have been included

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Contribution from the Snodgras Laboratory, St. Louis City Hospital Division, and the Department of Pathology, Washington University, School of Medicine, Saint Louis, Missouri.

TABLE I

DISTRIBUTION OF ANIMALS IN THE VARIOUS GROUPS

Stock diet		High fat diet					
		From 1 month through life	From 6 months through life	From 12 months through life	From 1 to 6 months	From 7 to 12 months	From 12 to 17 months
	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Total number of mice	100	76	61	39	65	51	49
Number of mice killed as scheduled	46	19	33	16	42	30	30
Number of mice dead or killed because of sickness and included in this report	54	57	28	23	23	21	19

(Table I). The most frequent anatomical cause of death was chronic kidney disease and anemia; occasionally liver tumors, leukemia, or pneumonia were observed.

Observations

Weights

The weights of mice fed the high fat diet from 1, 6, or 12 months on through life have been reported and discussed previously (5). The body weights of the animals receiving the high fat diet were in most cases higher than those of the stock-fed animals. The differences were the more marked the longer the fat-enriched diet had been consumed. In mice fed the fat-enriched diet for five months, the increase in mean weight was least striking, and of temporary character; it never exceeded 10% and was usually closer to 5% above that of the stock-fed animals. Mice fed the high fat diet from the age of 12 months on showed an only insignificant gain in weight. Following discontinuation of the high fat ration, the weights returned to normal and showed the usual decline with advancing age.

Life Span

In Table II, the mortality and the mean ages of the mice in the various groups at 100 days' intervals are presented, and in Table III, the mean and the maximum ages at which 25, 50, 75, or 100% of the animals were dead are shown.

TABLE II

MORTALITY OF ANIMALS OF THE VARIOUS GROUPS AT 100 DAYS' INTERVALS

	Stock diet	High fat diet					
	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Age, days	Mortality, %	Mortality, %					
300	2		4				
400	2	18	11		13		10
500	11	36	14	26	17		16
600	28	58	40	43	22	29	32
700	67	91	93	74	48	62	63
800	85	100	100	100	82	71	79
900	100				95	90	100
1000					100	100	

* *Since less than 100 animals were available in each group, only the full percentages are given.*

TABLE III

AGES AT WHICH 25, 50, 75, AND 100% OF THE ANIMALS WERE DEAD

Mice dead, %	Stock diet		High fat diet											
	Group I		Group II		Group III		Group IV		Group V		Group VI		Group VII	
	Age, days		Age, days											
	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean	Max.	Mean
25	557	481	438	382	540	424	486	433	606	456	584	541	538	458
50	664	559	541	447	663	507	628	491	709	554	656	587	646	524
75	717	603	637	449	696	567	713	557	719	607	817	635	743	584
100	874	653	730	546	701	600	722	593	972	655	993	694	879	643

Fig. 1 demonstrates the mortality curves of the mice of the various groups.

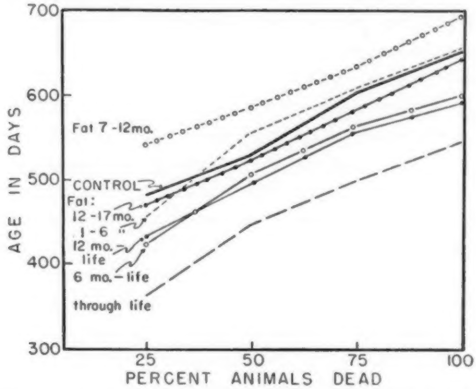

FIG. 1. Mortality curves of animals fed the stock or high fat diet for varying lengths of time.

Feeding of the High Fat Diet Throughout Life

The maximum age of mice fed the high fat diet on through life from the time of weaning (group II) was 144 days (16.1%) lower and the mean life span was 107 days (16.4%) shorter than that of the stock-fed animals (group I). Similarly in the group consuming the high fat ration, the 50% mortality was observed 112 days earlier than in the stock-fed control group.

Feeding the high fat diet from the ages of six months (group III) or 12 months (group IV) on and through life thereafter affected the life span of these animals less adversely than that of mice fed this ration from the time of weaning on (group II). Although in groups III and IV the maximum ages reached were 701 and 722 days respectively, that is, somewhat less than the 730 days attained by group II, the mean ages at death of all animals in groups III and IV were higher than in those of group II. As compared to stock-fed animals (group I), the mean life span of mice of groups III and IV was shortened by 8.1% and 9.2% respectively. Similarly, the median life span was 122 and 87 days longer in mice of groups III and IV than that of 541 days observed in group II, and in mice of groups III and IV, the 50% mortality was reached at a mean age of 507 and 491 days respectively as compared to a corresponding 447 days in group II. At the 25 and 75% levels of mortality, the same relations between groups II, III, and IV prevailed as at the 50 and 100% levels.

Feeding of the High Fat Diet for Five Months at Various Ages

The oldest individual of the group fed the fat-enriched diet from the age of one to six months (group V) outlived the oldest stock-fed mouse by 98 days. Otherwise the mortality of these mice was similar to that of the stock-fed animals. The high fat diet thus exerted no injurious effect on the life span of

mice of group V. In this series, however, the number of animals sacrificed on schedule was almost twice the number that died or were killed because of disease. When sacrificed, the animals were 18 and 24 months old respectively, the mean age being 591 days, that is 37 days higher than that of 50% of the mice of this group that died or were or killed because of sickness. Therefore, the mean life span of these mice might have been longer than indicated in the table, if all individuals had been allowed to live to the end of their natural lives.

Mice fed the high fat ration for five months from seven months of age on (group VI), lived somewhat longer than stock-fed controls (group I). This prolongation of life was associated with a decreased mortality as indicated by consistently higher mean ages at the 25, 50, 75, and 100% levels of mortality. This difference in age was approximately one month except at the 25% level, where it amounted to 60 days. A selective factor of minor importance may have favored these results. Animals started on the high fat diet at seven months of age were taken from a stock the early mortality of which was not determined. The elimination of a few individuals through death before seven months of age might somewhat decrease the mortality rate of the surviving animals, although this factor should not materially influence the results.

Animals fed the fat-enriched diet from the age of 12 to 17 months (group VII) had the least favorable survival. However, the differences between this group and groups V and VI were slight amounting to one month at the 50% mortality level and declining thereafter. The oldest age reached and the mean age of all mice were about the same as those of the stock-fed controls. The effect of the high fat diet in mice of this group may, however, be more injurious than would appear from the figures in the table. The selective factor mentioned before eliminates mice dead before one year of age and presumably leaves in the experimental group individuals of greater resistance and thus better survival than characteristic of the colony in general.

Discussion

Feeding a high fat diet from the ages of 6 to 12 months on through life shortened the life span of male mice of strain C57BL, but did so to a lesser degree than consumption of the same diet through life from the time of weaning on (6). There was no appreciable difference in the mortality of the mice receiving the diet from the ages of 6 or 12 months respectively. Yet, mice started on the high fat ration at the age of 12 months (group IV) consumed this diet for six months less than mice receiving the same ration from the age of six months on (group III). Thus there are indications that old mice are more susceptible to the injurious effect of the high fat diet than young adult mice.

Feeding the high fat ration for a period of five months had only minor effects on the life span of mice thus treated. The ration was slightly injurious only if fed to mice which were past the age of one year. Again thus, old animals tended to be most susceptible to the adverse effects exerted on the life span by a high dietary level of fat. If the high fat diet was given during

the period of growth and shortly thereafter, the life span was not altered, and if fed in early adulthood, the diet even proved beneficial.

The latter results agree with findings in rats in which a certain degree of dietary enrichment with butter fat was beneficial (3, 4) or innocuous (1) as far as longevity was concerned. However, there seems to be a limit either as to the level of fat in the ration or as to the length of time during which the high fat ration is given beyond which injurious rather than beneficial effects on life span will result from feeding such diets. The present observations together with those noted in mice underfed at various ages (2) suggest that age differences exist in regard to the susceptibility to dietary changes. Further investigations will have to define the limits within which high fat diets are beneficial to longevity and will also have to supplement the present data in regard to the possible role of age in determining the effects of diets on life span.

Summary

Feeding a high fat diet from the ages of 6 or 12 months on shortened the life span of male mice of strain C57BL, but was less effective in this respect than feeding of this ration from one month of age on. Old mice were more susceptible to the adverse effect on life span than young adult animals. The effect of feeding the high fat diet for five months at a time depended on the period of life during which the fat-enriched diet was consumed. Given during the period of growth, the high fat ration did not alter the life span; in young adults it had a beneficial effect, while given to old animals it exerted a slightly injurious action on the life span.

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CHROMATOGRAPHY OF STEROIDS PRODUCED BY RAT ADRENALS *IN VITRO*[1]

By F. H. Elliott and A. V. Schally

Abstract

As further evidence that rat adrenals *in vitro* produce corticoids, the chloroform-soluble substances formed by rat adrenals have been chromatographed on paper in two different solvent systems to compare their mobilities with those of known corticoids. The chromatography was carried out for the shortest possible time compatible with some resolution in order to prevent losses by excessive exposure or by transfer. Eluates of sections of the chromatograms were analyzed for α, β-unsaturated ketone groups and for groups that reduce a tetrazolium derivative (M. and B. 1767). The data may be interpreted as follows: 1. Rat adrenals *in vitro* produce ultraviolet-absorbing substances with chromatographic mobilities resembling hydrocortisone and corticosterone. Of these substances only 40–70% can be typical corticoids as judged by the reducing: ultraviolet-absorbing ratio. Ultraviolet-absorbing material without reducing properties is also present, most of which has a polarity greater than corticosterone. 2. ACTH, added to rat adrenal glands *in vitro*, stimulates primarily the production of substances with a polarity similar to that of corticosterone; the production of the other components is also somewhat increased.

Introduction

In previous papers from our laboratory (11, 12, 7) substances formed by rat adrenals *in vitro* were shown to have the following properties:

1. They are extractable by chloroform.
2. They absorb ultraviolet light, with a maximum at 240 mμ.
3. They reduce a substituted tetrazolium reagent, M. and B. 1767.
4. Their production is increased by ACTH.
5. The increased production is dependent upon the amount of ACTH added.
6. They decrease the number of circulating eosinophiles in adrenalectomized mice.
7. They are not formed by testis and liver tissue.

These properties describe those of the typical corticoids so that it was concluded that the rat adrenal produces corticoids *in vitro* (11). To strengthen this conclusion, the substances formed by rat adrenals *in vitro* were chromatographed on paper in two different solvent systems to compare their mobilities with those of known corticoids. The chromatographic separations served, in addition, to localize the substance(s) found by Elliott *et al.* (7) which absorbs light at 240 mμ, but which does not reduce the substituted tetrazolium compound, M. and B. 1767.

The chromatography of compounds formed by rat adrenals *in vitro* was carried out for a relatively short period so that the main constituents did not move off the paper strips and so that they would be exposed for the shortest possible time compatible with some resolution. Under these conditions not all the constituents separate, but a division into three classifications of material

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Contribution from the Allan Memorial Institute, McGill University, Montreal, Quebec. This work was aided by Federal Provincial Mental Health Grant Nos. 604-5-12 and 15 combined to Dr. R. A. Cleghorn.

was achieved along with good recoveries of the total original material. A comparison of the location of the experimental material with that of 17-hydroxycorticosterone, corticosterone (Kendall's compounds F and B), and desoxycorticosterone (DOC), chromatographed at the same time, was used as the only method of classification.

Methods

Ten or twelve bisected rat adrenals were incubated for two or three hours and the medium was extracted with chloroform, as previously described (7). Portions of the chloroform extract were analyzed for α,β-unsaturated ketonic groups by the ultraviolet absorption method (UV method) and for α-ketolic groups by using a triphenyl tetrazolium chloride derivative, M. and B. 1767 (MB method (7)).

The residue from the remaining extract was chromatographed by the toluene – propylene glycol system of Burton, Zaffaroni, and Keutmann (2) or with a more convenient solvent system similar to that of Bush (3), which we developed. This consists of the upper phase of a mixture of 1.5 volumes of heptane and 1 volume each of toluene, methanol, and water. The material in chloroform solution is applied to dry paper strips that had been previously washed by descending chromatography with methanol for two or three days. The strips are developed with the solvent mixture at room temperature, 26°–27°, for 24 hr. When this system is used, the known corticoids separate in the same order but at a somewhat faster rate than with the Burton, Zaffaroni, and Keutmann system. The drying time after chromatography is greatly reduced.

The ultraviolet-absorbing substances on the paper strips were located by the ultraviolet fluorescence scanner (9) or by scanning in the Beckman spectrophotometer (14). The spots were eluted with three 1-ml. portions of methanol and were analyzed as above.

The reducing substances on the paper can be located with a solution of M. and B. 1767 in sodium hydroxide (2 ml. of a 0.1% solution of M. and B. 1767 in 95% ethanol and 5 ml. of 2.5 *N* sodium hydroxide). The strip is dipped into this solution immediately after the reagents are mixed. A deep purple color develops with 10 μgm. of each of the six corticoids and a very weak purple color with 1 μgm. Practically no color develops with 20 μgm. of progesterone.

Errors of Measurement

Methanol solutions of material eluted from papergrams contain impurities that come from various sources, including the paper itself. The error that these impurities introduce in the determination of steroids by ultraviolet absorption can be evaluated from the ultraviolet absorption curves of these methanol solutions using calculated densities, CD_1 and CD_2, as described previously (7): $CD_1 = OD_{240} - \frac{1}{2}(OD_{225} + OD_{255})$; $CD_2 = OD_{240} - OD_{260}$. The difference between CD_1 and CD_2 has been shown to be a measure of the distortion of the absorption curve due to impurities.

TABLE I

CD_1, CD_2 DIFFERENCES OF METHANOL ELUATES OF CHROMATOGRAPHED MATERIAL

	Development time of the chromatography	
	4 to 7 hr.	18 to 30 hr.
	μgm. steroid	
Material chromatographed:		
Compounds F, E, and B	1.0 ± 0.9 (S.D.) (26)*	2.6 ± 2.2 (12)
DOC	3.8 ± 2.9 (13)	—
Material from extracts of adrenal incubates† with R_fs of:		
Compounds F, E, and B	3.4 ± 2.2 (23)	2.7 ± 2.0 (27)
DOC	23.0 ± 12.8 (8)	—

* *Number of tests.*
† *Media in which the adrenals had been incubated two or three hours after a preincubation period. Preincubation media contain more impurities in all sections of their chromatograms than these incubation media.*

In Table I are shown the CD_1, CD_2 differences of methanol solutions of steroids and of material from paper chromatograms of chloroform extracts of adrenal incubation media. Since the steroid value is calculated from the average of the CD_1 and CD_2 values, the error that the impurities introduce is $\frac{1}{2}$ of the CD_1, CD_2 difference. Thus for compounds F, E, and B, after four to seven hours chromatography, the average error is ± 0.5 μgm. steroid per section and after 18 to 30 hr. chromatography, ± 1.3 μgm. The average error involved in the recovery of DOC is higher, ± 1.9 μgm., since there is a large amount of impurity near the solvent front where DOC is found. For material from adrenal incubation media which has chromatographic mobilities like those of compounds F, E, and B, the average error is ± 1.7 μgm. for the four to seven hour chromatography and ± 1.4 μgm. for the longer chromatography. The fast moving material with the high CD_1, CD_2 difference is probably mostly impurity.

In Table II are shown the per cent recoveries of Kendall's compounds F, E, and B and DOC after chromatography as determined by the UV and the MB methods. It is seen that the chromatographic loss is relatively small for both the short and the long periods of chromatography. The longer period of chromatography was used in the experiments to be discussed later since it permits a better separation of the constituents.

Attempts were made to measure the corticoids directly on the paper strips according to the method of Tennent *et al.* (14). However, the presence of ultraviolet-absorbing impurities on the paper rendered such measurements inaccurate, especially at both ends of the paper strips.

TABLE II

RECOVERY OF KNOWN STEROIDS AFTER CHROMATOGRAPHY

Steroids	Development time, hr.	% recovered	
		By UV method	By MB method
Compounds:			
F, E, and B (10–23 μgm. each)	4–7	83.7 ± 8.5 (S.D.) (24)*	85.5 ± 7.5 (24)
F, E, and B (9–14 μgm. each)	18–30	85.1 ± 12.0 (12)	96.0 ± 15.5 (6)
DOC (7–13 μgm.)	4–7	95.0 ± 13.0 (13)	81.0 ± 11.2 (12)

* *Number of tests.*

Experimental Results and Discussion

Figs. 1, 2, and 3 illustrate typical experiments in which known steroids and chloroform extracts of adrenal incubation media with and without ACTH were chromatographed at the same time. Chromatographic losses were very small because the sum of the values obtained by the UV and by the MB method, of all sections of a chromatogram, was practically the same as the value obtained for that extract before chromatography. From the absorbance curves obtained by direct scanning of the paper and from the elution values, shown by bars, it is seen that most of the UV-absorbing compounds as well as α-ketol compounds present in incubation media have chromatographic mobilities equal to or less than Kendall's compound B. Since the glands incubated with ACTH and those incubated without ACTH were paired and weighed nearly the same, the data from the two chromatograms containing incubation material in each of the three experiments are practically comparable. It can be seen that there are nearly equal amounts of "F" and "B" material in the incubation media without ACTH and that the effect of ACTH was to increase the formation of "B" material, both UV-absorbing and α-ketolic, and in experiment 1 (Fig. 1) to increase also the production of "F" to "B" material, which is UV-absorbing but not α-ketolic.

From the results of the above experiments and of other similar ones it is possible to make the following generalizations about the steroid production of Sprague-Dawley rat adrenals incubated under the conditions used:

a. An average of 80% of the substances measured by the MB method and by UV absorption in adrenal incubation media has chromatographic mobilities equal to or less than Kendall's compound B. The UV-absorbing, non-α-ketolic substances, represented by white sections of the bars in Figs. 1, 2, and 3, are largely recovered in the "F" through "B" sections of the chromatograms.

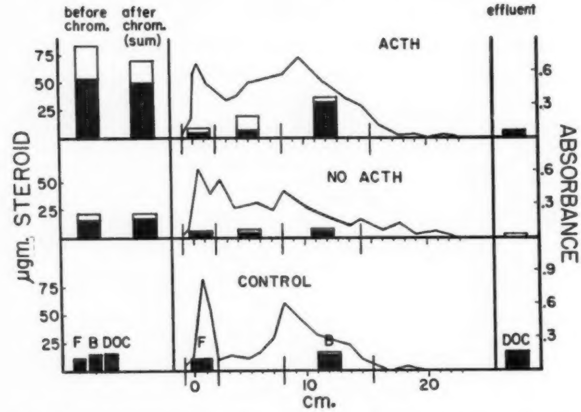


FIG. 1. Absorbance curves and elution values of chromatographed extracts of adrenal incubation media.

The height of each bar represents μgm. of steroid measured by the UV method. The height of the black section of each bar is the steroid value by the MB method. The paper sections indicated by long vertical lines on each abscissa were cut, eluted, and analyzed by the two methods. The eluate that collected during the development time was also analyzed and these results are shown by bars labelled "effluent". The values before chromatography, represented by the bars at the left, are the amounts of material put on the starting lines of the papers. The bars labelled "sum" show the sum of the values of the eluted sections and effluent. None of the values for chromatographed material have been corrected for chromatographic loss. Solid curves represent the absorbance readings obtained by scanning the papergrams in a Beckman spectrophotometer.

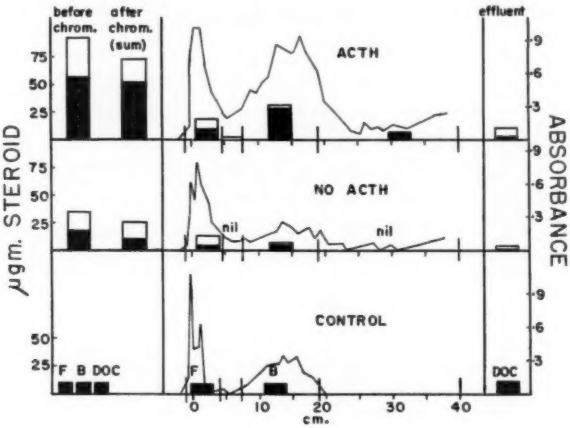
The development time was 30 hr. and the solvent system used was toluene – propylene glycol.

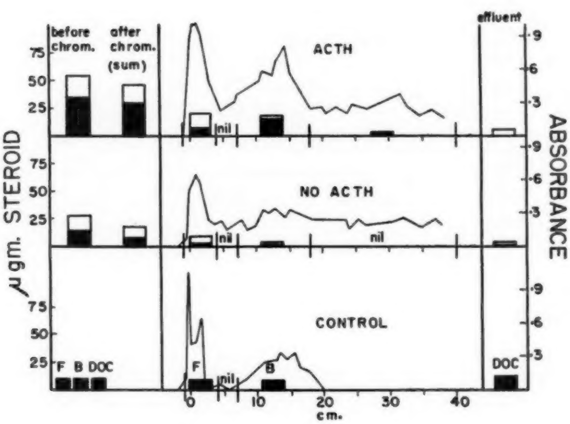
b. The material represented by the MB values of Section "B" has the mobility of Kendall's compound B and constitutes one third to one half of the total UV-absorbing substances found after incubation with ACTH. It is also the constituent whose formation is stimulated to the largest extent by ACTH.

c. The formation of the material represented by MB values in Section "F" is stimulated by ACTH to a much smaller extent than the formation of the "B" substances. These two findings (*b* and *c*) agree with the reports of Bush (3, 4, and 5) that the rat adrenal stimulated by ACTH secretes mainly Kendall's compound B and very little, if any, compound F.

d. The UV-absorbing, non-α-ketolic substances have been found in all sections but they seem to be concentrated in the "F" section (experiments 2 and 3) or in the "F" to "B" section (experiment 1). There is the possibility that there may be more than one UV-absorbing, non-α-ketolic steroid present. Their amounts in each section are too small to be reliable so that stimulation of their formation by ACTH can only be inferred from the sum of their values.

Apart from Kendall's compounds F and B that have been found as the main cortical steroids in adrenal vein blood, peripheral blood, and adrenal perfusates of various species, other steroids have been detected that are α-ketolic and without a UV-absorbing group (10, 13, 8). If there are any such α-ketolic,





FIGS. 2 and 3. (See Legend of Fig. 1.) The development time was 24 hr. and the solvent used was the upper phase of a mixture of heptane, toluene, methanol, and water = 3 : 2 : 2 : 2.

non-UV-absorbing steroids present in rat adrenal incubation media, then the amount of the UV-absorbing, non-α-ketolic steroids present is greater than we have assumed.

Brady (1) has found in incubation media of dog adrenal slices a UV-absorbing, non-α-ketolic component with a polarity somewhat less than that of Kendall's compound E. It is possible that this steroid reported by Brady is of the same type as the material indicated by our data. Bush (6) also found a UV-absorbing, non-α-ketolic component in the adrenal vein blood of rats, which he believes to be 11-hydroxyandrost-4-ene-3,17-dione. This steroid has a polarity less than that of compound B. If this 17-ketosteroid is present in rat adrenal incubation media, it does not account completely for our findings.

Acknowledgments

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ZINC, CARBONIC ANHYDRASE, AND PHOSPHATASE IN THE PROSTATIC GLANDS OF THE RAT[1]

By M. I. Fischer, A. O. Tikkala[2], and C. A. Mawson

Abstract

The concentration of zinc in the dorsolateral prostate of the rat increases rapidly during infancy, reaching a maximum of 250-300 μgm./gm. at an age of about 160 days. This figure is 10 times as high as that found for most other soft tissues. Carbonic anhydrase activity behaves similarly, with a maximum at about 200 days, when it is 100 times the amount found in most other organs. Zinc which occurs as part of the carbonic anhydrase molecule forms only a small proportion of the total zinc content of the tissue at all ages. Zinc and carbonic anhydrase occur only in small amounts in the ventral prostate. The activities of acid and alkaline phosphatases in ventral and dorsolateral prostates are not correlated with age, and zinc and enzyme concentrations bear no consistent relationship to one another. Differences between the phosphatases of liver and prostatic complex in the rat are briefly discussed.

It has been suggested that the zinc found in tissues might be concerned in some way with the synthesis of enzymes or of proteins in general. The dorsolateral prostate of the rat contains a high concentration of zinc while the ventral prostate contains very little. The two prostates are histologically very similar and if the synthesis of enzymes were dependent upon the presence of zinc they might exhibit related differences in enzyme activity, particularly if zinc and enzyme concentration were followed from an early age to maturity. In the present work carbonic anhydrase and the acid and alkaline phosphatases have been estimated in rat prostates at various ages, and results compared with the corresponding zinc concentrations of the glands.

Methods

Tissues were obtained from Collip hooded rats which were killed by bleeding.

Zinc was estimated by the method of Vallee and Gibson (15) after the tissue had been dried in platinum dishes and ashed at 520°.

Carbonic anhydrase was estimated in diluted homogenates by the colorimetric method of Roughton and Booth (11). This method is extremely sensitive to temperature change and it was essential to work in a cold room at 4°. The reaction was observed in 20 ml. stoppered weighing bottles which stood side-by-side against the wall of a glass staining dish packed with finely-crushed ice.

Acid and alkaline phosphatase were determined in tissue homogenates by a modification of the methods used by Abul-Fadl and King (1). Results are expressed per gm. wet weight in the units used by King and his collaborators—i.e. mgm. phenol liberated per hour for acid phosphatase and mgm./15 min. for alkaline phosphatase.

Histological staining of zinc was carried out in tissues fixed in 90% methanol – 10% formalin by the method of Okamoto (6).

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Results

Zinc in Dorsolateral Prostate at Different Ages

The dorsolateral prostates of newborn rats were too small for accurate dissection, and at age 40 days it was necessary to pool the glands from eight rats to obtain sufficient tissue for analysis. Six rats were used at 47 days and four at 54 and 66 days. The remaining zinc analyses were performed on pooled prostates from three rats.

The results are shown in Fig. 1. The concentration of zinc in the prostate tissue increased rapidly from infancy to an age of about 160 days, and then decreased. The peak of the curve corresponds with the region in which the growth-curve of the rats in our colony levels off (Fig. 2). The curve relating prostate weight to age (Fig. 3) also turns at about this point.

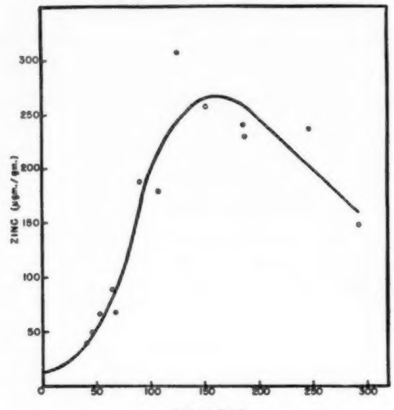
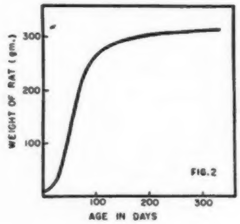


FIG. 1. Concentration of zinc in the dorsolateral prostate of the rat at different ages. (The curve is compounded from $f_1(x) = 10.8506 + 0.99054.10^{-2}x + 1.6539.10^{-2}x^2$ for $0 \leqslant x < 75$ and $f_2(x) = -3.8750.10^2 + 9.7083 \times -4.5115.10^{-2}x^2 + 6.2633.10^{-5}x^3$ for $85 < x < 280$.)



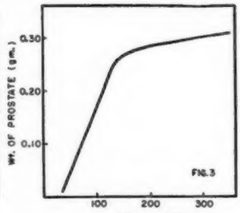


FIG. 2. Weight of the Collip hooded rat at different ages. Mean curve plotted from weights of 93 animals.

FIG. 3. Weight of dorsolateral prostate gland at different ages. Mean curve plotted from prostates of 86 animals.

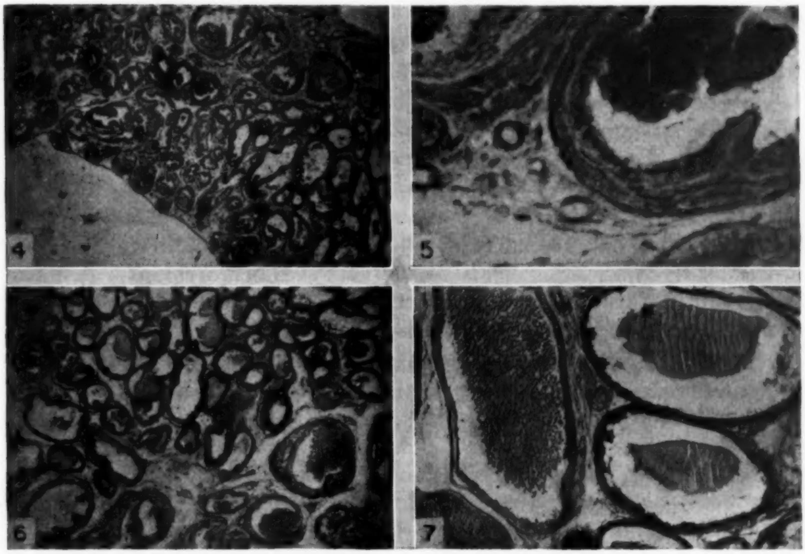


FIG. 4. Age 50 days. × 13.5. Scattered zinc-positive acini; most of tissue unstained.

FIG. 5. Age 50 days. × 152. All cells in acinus not equally stained. Most intense stain in tissue within acinus.

FIG. 6. Age 63 days. × 13.5. Upper part stained positively for zinc, lower part unstained. Some acini within the stained (lateral) portion of the gland remain unstained.

FIG. 7. Age 67 days. × 38. Sharply defined difference between positive-(lateral) and negative-(dorsal) staining areas. The positive-staining area is fully developed and all acini are stained.

Photomicrographs taken with green filter of dorsolateral prostates of rats aged 50 to 67 days. Stained with Okamoto zinc-specific stain—positive reaction for zinc is intense red, reproduced in photographs as black areas, contrasting with negatively reacting tissue which is unstained or pale salmon-pink. Direct illumination—Leitz Ortholux microscope.

Specific Zinc Stain

Our male rats became able to breed between the 50th and 55th day, and it is at this point that the first positive results were obtained with the Okamoto zinc stain.

A few red-staining areas were found within isolated acini in the lateral ends of the dorsolateral prostate at about the 50th day, and by the 55th day scattered acini were found in which all the cells reacted positively. By the 60th day numerous zinc-staining acini were present throughout the lateral glands (Fig. 4, 5, 6 and 7).

The location of the zinc-staining areas showed that it would be desirable to analyze these parts separately from the dorsal portion. It was, however, difficult to distinguish the lateral lobe of the gland in fresh tissue from adult rats, and in young rats it was impossible to decide, even approximately, where the gland should be divided, so no attempt was made to perform separate analyses on these tissues at different ages.

Ventral Prostate

The zinc content of the ventral prostate of the adult rat is so small (8) that analyses of this organ at different ages were not made. The Okamoto stain gave negative results.

Carbonic Anhydrase in Prostate at Different Ages

Carbonic anhydrase was estimated in the prostates of rats aged 34–348 days. Pooled tissue from five glands was used at 38 days, from two at 52 days and from one at 286, 292, 309, 318, and 348 days. Three glands were used in all the other experiments. The tissue was homogenized with water in a glass homogenizer and diluted with peptone-water. The final concentration of peptone, which was used as a stabilizer (13), was 0.05%.

Preliminary investigation showed that homogenization and dilution either with water or physiological saline gave the same results for carbonic anhydrase in ventral prostate whether the whole homogenate or the centrifuged extract was used, but there seemed to be a slight advantage in using the whole water homogenate of the dorsolateral prostate. The final dilution of tissue added to the buffer–substrate mixture in the carbonic anhydrase estimation was 1/100 for ventral prostate and 1/7500–1/10000 for dorsolateral prostate.

Carbonic anhydrase activity in ventral prostate was low at all ages (0.05–0.22 units/mgm. wet wt.) and showed no correlation with age. In the dorsolateral prostate the results were rather scattered in the lower age groups but there was a trend towards higher enzyme content with increasing age up to about the 190th day, followed by a decline (Fig. 8).

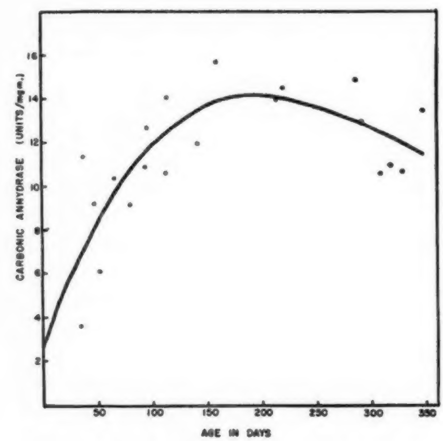


FIG. 8. Activity of carbonic anhydrase in the dorsolateral prostate of the rat at different ages. (The curve is given by
$f(x) = 2.603 + 0.1375x - 5.025.10^{-4}x^2 + 5.200.10^{-7}x^3$.)

Phosphatases in Prostates

In the method for the estimation of phosphatases described by Abul-Fadl and King (1), the tissues were ground with sand and mixed with an equal weight of physiological saline plus 10% v/v of a mixture of equal parts of toluene and ethyl acetate. After autolysis for two or three days at room temperature and centrifugation, the supernatant liquid was filtered, diluted with saline, and tested for phosphatase activity. This extraction procedure was inconvenient for small amounts of tissue, and preliminary experiments showed that the highest enzyme activities were given by whole homogenates made with 10 volumes of water and tested immediately after preparation. This was the standard procedure in our work on phosphatase activity at different ages, but the preliminary experiments gave results of sufficient interest to be briefly described. The results of the exploratory investigation are given in Tables I and II.

TABLE I

ACID PHOSPHATASE IN RAT PROSTATE COMPLEX—EFFECT OF DIFFERENT EXTRACTION METHODS

(Phosphatase in King-Armstrong units/gm. wet weight of tissue. Homogenates prepared and diluted in distilled water or 0.85% w/v sodium chloride)

Tissue	Rat No.	Homogenate		Supernatant		Deposit	
		Water	Saline	Water	Saline	Water	Saline
Dorsolateral	1	7.6		2.8		4.2	
prostrate	2		2.9		1.6		1.2
	3	10.5	8.5	2.6	2.8		
Ventral	6	16.0	13.9	12.6	9.4		
prostate	7	13.0		6.8		4.9	

TABLE II

ALKALINE PHOSPHATASE IN RAT PROSTATE COMPLEX—
EFFECT OF DIFFERENT EXTRACTION METHODS

(Phosphatase in King-Armstrong units/gm. wet weight of tissue. Homogenates prepared and diluted in distilled water or 0.85% w/v sodium chloride. When Mg^{++} was added the final concentration was 0.0088 *M*)

Tissue	Rat No.	Added Mg^{++}	Homogenate		Supernatant		Deposit	
			Water	Saline	Water	Saline	Water	Saline
Dorsolateral	4	0	18.6		1.6		15.4	
prostate	4	+	44.6		5.0		33.0	
	5	0		18.3		9.3		4.3
	5	+		40.0		17.7		13.1
Ventral	8	0	62.6		36.0		18.0	
prostate	8	+	84.4		47.4		25.9	
	9	0		75.6		66.0		3.8
	9	+		98.8		91.4		4.2
	10	0	40.7	37.6				
	11	+	93.4	90.6				

Comparisons were made between the phosphatase activities of homogenates of dorsolateral and ventral prostates. The enzyme activity of the whole homogenate was compared with that of the supernatant and deposit obtained by centrifugation for 10–15 min. at 2000 r.p.m. in an International Clinical Centrifuge. Supernatants from dorsolateral prostate were clear, pH about 6.5, while those from ventral prostate were somewhat opalescent, pH about 7.0. Deposits were washed twice before dilution to the original volume of the homogenate. Results of the experiments are summarized below and in Tables I and II.

(1) The optimum pH of acid phosphatase from both prostates was 5.5, using citrate buffer. The alkaline phosphatase of both tissues had optimum pH 9.8–10.0 in carbonate–bicarbonate buffer.

(2) Acid and alkaline phosphatases were stable at $-15°$ but lost activity on standing at 6°. With ventral prostate homogenate the mean losses of acid phosphatase after one, two, four, and six days at 6° were 17, 25, 37.5, and 39.5%. Addition of toluene did not prevent loss of activity.

(3) Physiological saline extracted the enzymes more efficiently than water, but the presence of sodium chloride in the whole homogenates slightly depressed their activity. Nearly all the alkaline phosphatase of ventral prostate was soluble in saline but much less was extracted by water. Much of the acid phosphatases of both glands, and of the dorsolateral alkaline phosphatase, were not removed from the deposit either with water or saline.

(4) Addition of Mg^{++} to homogenate, supernatant, or deposit from either gland increased the activity of alkaline phosphatase. The optimum concentration of added Mg^{++} was 0.0088 *M*.

Effect of Age on Phosphatases of Prostates

Acid and alkaline phosphatases were estimated in ventral and dorsolateral prostates at ages 39–318 days (Table III). No correlation was observed between age and phosphatase activity. Phosphatase behaved quite differently in this respect from carbonic anhydrase.

Discussion

In evaluating the relationship between the zinc content of tissues and their enzyme activity several possibilities must be considered. Zinc may form part of the enzyme molecule, as it does in carbonic anhydrase (7), or may be an activator of the enzyme without being an intramolecular constituent, as suggested for phosphatase by several authors quoted by Abul-Fadl and King (2), and Sadisavan (12). The metal may be unconnected with the enzyme once the latter has been synthesized but be essential for some process involved in enzyme formation (10). It is difficult to interpret results obtained from tissues in which the zinc- or enzyme-containing cells may form only a small proportion of the whole, and more than one of the above possibilities may operate at the same time. However, it was interesting to compare

TABLE III

PHOSPHATASES IN RAT PROSTATE COMPLEX—VARIATION WITH AGE

(Homogenates prepared and diluted in water. Phosphatase in King-Armstrong units/gm. wet weight of tissue)

Age of rats (days)	Ventral prostate			Dorsolateral prostate		
	Acid phosphatase	Alkaline phosphatase		Acid phosphatase	Alkaline phosphatase	
		No Mg^{++}	0.0088 *M* Mg^{++}		No Mg^{++}	0.0088 *M* Mg^{++}
39	14.1	45.6		12.6	12.2	
46	14.1	68.4		11.8	27.1	
54	14.3	99.2		9.6	15.6	
65	11.8	46.9		6.6	20.5	
82	13.2	51.8		10.2	34.2	
98	14.7	44.9		8.3	23.4	
102				11.7	21.5	
104				10.1	14.6	
114	17.7	44.1				
117	13.9					
139			81.9			33.8
148	17.8	57.4				
154	14.4		107.2	11.4		34.8
170	15.0		76.8	14.7		45.7
172			66.0			41.2
172	15.2		78.0	10.2		24.7
174	17.1		65.8	15.4		41.7
185			98.8			40.0
190		40.7	77.0		19.8	40.2
192			93.4			29.2
318	9.4	39.5				

the rate of accumulation of zinc in the prostates of the young rat with the amount of phosphatase and carbonic anhydrase in these glands at various ages. In the adult rat a very high proportion of prostatic tissue consists of secreting acini, and although the ventral prostate is histologically very similar to the dorsolateral gland the zinc contents of these two tissues are very different (Table IV). The dorsal and lateral parts of the dorsolateral gland are almost indistinguishable histologically, but they again contain different amounts of zinc.

It has been shown that the zinc concentration of the dorsolateral prostate was not much above that of most other tissues at age 40 days but rose rapidly to a maximum of 250–300 μgm./gm. at about 160 days. A high proportion of the zinc was in the lateral lobes, but accurate figures for variation with age were unobtainable in this tissue owing to the difficulty of distinguishing the dividing line between the dorsal and lateral organs. Beyond 160 days

TABLE IV

ZINC, CARBONIC ANHYDRASE, AND PHOSPHATASES IN PROSTATE COMPLEX OF THE RAT—SUMMARY OF RESULTS

(Phosphatase results in columns 2 and 3 are means from Table III. Remaining results are means from rats aged over 100 days. Alkaline phosphatase estimated without added Mg^{++})

Organ	Ventral	Dorsolateral	Dorsal	Lateral
Zinc (μgm./gm.)	13.7	180	103	590
Carbonic anhydrase (units/mgm.)	0.11	10.5	5.1	16.0
Acid phosphatase (units/gm.)	14.8	11.1	9.6	12.4
Alkaline phosphatase (units/gm.)	53.9	21.0	16.5	23.6

Values for zinc in ventral and dorsolateral prostate from Mawson and Fischer (8).
Values for carbonic anhydrase from Mawson and Fischer (9).

the zinc concentration of the whole gland decreased. The zinc concentration of the ventral prostate showed no comparable variation and remained low at all ages.

The carbonic anhydrase activity in the dorsolateral prostate increased with advancing age to a maximum at about 200 days, followed by a decline. Variations between animals prevent accurate location of curves of this nature but it seems fair to say that although the general trends of the zinc and carbonic anhydrase curves are similar they are not closely correlated. Keilin and Mann (7) found that ox carbonic anhydrase contained 0.3% zinc as an obligatory part of the molecule and we have observed that it has a dry weight of 0.2 μgm./enzyme unit. Assuming identity of ox and rat carbonic anhydrase, the amount of zinc directly associated with the enzyme in the mature rat dorsolateral prostate is about 6.3 μgm./gm. tissue, which is only 3.5% of the total zinc content of 180 μgm./gm. This contrasts with the rat erythrocyte, where we have found a mean zinc concentration of 11.6 μgm./gm. and a carbonic anhydrase activity of 10.4 units/mgm. Here, 54% of the total zinc was contained in carbonic anhydrase.

The prostate is normally engaged in secretion and cell-replacement and is capable of rapid regression and regeneration, and might be expected to have stores of essential material for synthesis of an important enzyme. The erythrocyte has no comparable synthetic activity but its carbonic anhydrase is constantly in action, and if this led to accumulation of inert zinc-containing breakdown products they would be expected to be found in the erythrocyte. We know from unpublished turnover experiments with Zn^{65} that the zinc of the dorsolateral prostate is in a fairly mobile form which is unlikely to be waste material from decomposed carbonic anhydrase. This is supported by the observation that a very large excess of zinc over that in carbonic anhydrase is present in the gland at all ages.

Very high concentrations of zinc in tissues are not always necessary for the formation of carbonic anhydrase. Rat pancreas contains only about 23 μgm. Zn/gm. wet weight, yet the carbonic anhydrase concentration is stated to be as high as that of blood (5). The lens of the eye, according to Bakker (4) contains a high concentration of carbonic anhydrase and Tauber and Krause found a considerable amount of zinc in the same tissue (14) whereas sperm, which contain a very large amount of zinc, have only a trace of carbonic anhydrase activity (10). It seems, therefore, that the apparent correlation observed between metal and enzyme during development of the rat dorsolateral prostate cannot be assumed to be directly related. It is known (10) that zinc is present in human prostatic secretion, and unpublished autoradiographic evidence suggests that this is also true of rat secretion, but the possibility still remains that some as yet unrecognized synthetic activity within the prostatic cells requires a high concentration of zinc.

The formation of phosphatase does not require the presence of more zinc than was found in the prostates of the youngest rats examined. In mature rats both acid and alkaline phosphatase had higher activity in the ventral than in the dorsolateral prostate, while the zinc concentration showed the reverse relationship. There was no consistent trend in acid or alkaline phosphatase activity during development in either gland.

The properties of the phosphatases of rat prostates differ somewhat from those investigated in the intracellular fractions of rat liver by Allard *et al.* (3). We did not attempt to separate intracellular fractions, but our supernatants contained no obvious particulate material. Allard *et al.* found that alkaline phosphatase activity of liver supernatant was only measurable in presence of Mg^{++} whereas the enzyme attached to the deposit (one-fifth of the whole) was not activated by Mg^{++}. Alkaline phosphatase of dorsolateral prostate (Table II) occurs mainly in the deposit under our conditions of extraction and the enzyme in all fractions was activated by Mg^{++}. Alkaline phosphatase of ventral prostate was present in higher proportion in supernatant than in deposit, and again all fractions were activated by Mg^{++}.

Acid phosphatase of liver occurs mainly in the deposit, but in both prostates it was about equally divided between supernatant and deposit. (Table I).

Allard *et al.* noticed that a proportion of both phosphatases was present in liver in an inactive form which became active after 60 min. incubation at 37°. We did not preincubate our tissues or extracts at 37°, but their activity decreased, rather than increased, on standing at room temperature or at 6°.

Acknowledgments

This investigation was commenced as a result of unpublished work by Miss B. P. Clayton on histochemical localization of phosphatases in rat prostates. The histology in the present work has been carried out by Mr. C. M. Gravelle and the photographic processing by Mr. N. R. Vincent. We are greatly indebted to Miss P. Dyson for fitting curves to the experimental data.

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AN ASSAY METHOD FOR LIPOXIDASE IN ANIMAL TISSUE[1]

By D. H. J. Boyd[2] and G. A. Adams

Abstract

An assay for lipoxidase in animal tissues has been developed which eliminates the interference of heme compounds. Catalytic oxidation of linoleate emulsions at pH 9.0 by heme pigments was inhibited by potassium cyanide while lipoxidase activity was relatively unaffected. The assay method was capable of detecting extremely low levels of lipoxidase activity in animal tissue. Application of the method to beef and pork adipose tissue, uncured bacon, cured unsmoked bacon, and rabbit liver, kidney, spleen, heart, brain, and lung strongly suggested that lipoxidase was not present in these tissues and that the linoleate oxidation was catalyzed by the heme pigments in the extracts.

Introduction

The enzyme lipoxidase, which catalyzes the oxidation of unsaturated fatty acids, is found in plant tissues but its presence in animal tissues has not been demonstrated unequivocally. In some reports (11, 12, 15, 9), the oxidative activity of animal tissues towards unsaturated fats has been attributed to lipoxidase; in others (2, 3, 22), to heme compounds. Tappel (17, 19) has recently reported a method differentiating between heme and lipoxidase based on differences in their ability to oxidize sodium linoleate substrates at pH 7.0 and pH 9.0. Using this assay, he found no lipoxidase activity in a variety of animal tissues.

Because of the conflicting results reported by other workers, a new method of assaying lipoxidase activity in the presence of heme compounds has been developed in this laboratory. The procedure was suggested by the findings of Khan (9) and Barron *et al.* (4) that cyanide inhibits heme activity and of Süllman (14) and Holman (8) that potassium cyanide has no inhibiting effect on lipoxidase activity.

Materials and Methods

Preparation of Substrate—Sodium Linoleate

Sodium linoleate emulsions (0.045 *M*) were used in these experiments. The linoleic acid was a highly purified product obtained from the Hormel Institute, Austin, Minn. The acid (128 mgm.) was neutralized with sodium hydroxide, shaken thoroughly, and the resulting suspension was diluted with borate buffer (pH 9.0) to a final volume of 10 ml. To obtain reproducible figures for gas uptake, the sodium linoleate emulsions were prepared just prior to use.

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[2] *National Research Council of Canada Postdoctorate Fellow, 1953–54.*

Preparation of Lipoxidase Solution

Soybean lipoxidase obtained from Mann Research Laboratories Inc., New York, was dissolved in borate buffer of pH 9.0 to form a 0.05% solution. This solution showed a lipoxidase activity of 1.2 units per ml., as assayed by the method of Bergström and Holman (5).

Preparation of 2 M Cyanide Solutions

Potassium cyanide (0.652 gm.) was dissolved in water and the solution adjusted to pH 9.0 with concentrated hydrochloric acid. Borate buffer of pH 9.0 was then added to a volume of 5.0 ml. These solutions were prepared just prior to use.

Measurement of Substrate Oxidation

The Warburg respirometer was used throughout to follow the course of linoleate oxidations. Tappel (17) showed that valid determinations of the rate of oxygen uptake by linoleate could be obtained by this method. For oxygen absorption measurements, unless otherwise stated, 1.0 ml. of sodium linoleate (0.045 *M*) was placed in the sidearm, with the catalysts and inhibitor (added in that order) in the main part of the vessel. The total volume of liquid in the vessels was brought to 3.0 ml. by borate buffer (pH 9.0) where-ever necessary. The manometers and vessels were gassed with oxygen for 10 min. and then equilibrated for 10 min. prior to tipping in the substrate. In experiments where extracts of rabbit tissue were being studied, potassium hydroxide was placed in the well to absorb respiratory carbon dioxide. All experiments were carried out at 18.8° C. and control experiments which permitted correction for auto-oxidation of the substrate were included.

Preparation of Tissue Extracts

The animal tissues investigated were hog adipose, beef adipose, uncured bacon, cured unsmoked bacon, and rabbit liver, kidney, spleen, heart, brain, and lung. Pork and beef tissues were diced and then disintegrated in a Waring blendor with an equal weight of borate buffer (pH 9.0). The extracts obtained were centrifuged at 780 $\times$ *g* for 20 min. and the supernatant liquors were further clarified by spinning at 11,180 $\times$ *g* again for 20 min. Clear, amber-colored supernatants containing the active catalysts were obtained. These extracts were always prepared just prior to use. One milliliter of extract was equivalent to 3–5 gm. of whole tissue.

Fresh rabbit tissues were homogenized with an approximately equal volume of borate buffer (pH 9.0) in a Potter-Elvehjem homogenizer. The resultant homogenates were centrifuged for 30 min. at 80,000 $\times$ *g*. The clear supernatant extracts thus obtained were usually a pink or red color. When the red color was pronounced, it was necessary to make appropriate dilutions with buffer solution. One milliliter of the extracts was equivalent to 1–3 gm. of intact tissue.

Preparation and Assay of Heme-containing Compounds

Hemoglobin solutions were prepared from hog blood using the method of Urbain and Jensen (21) and were assayed by conversion to methemoglobin and spectrophotometric analysis (1).

Crystalline myoglobin and metmyoglobin were prepared from horse heart using the method of Theorell (20) and were assayed by the spectrophotometric method of de Duve (6).

Purified cytochrome-*c* of known concentration and crystalline catalase were obtained from General Biochemicals Inc. Crystalline hemin was obtained from Nutritional Biochemicals Corp.

Experimental and Results

The Oxidation of Sodium Linoleate Catalyzed by Soybean Lipoxidase

The catalytic oxidation of sodium linoleate (0.015 *M*) by soybean lipoxidase was studied over a range of enzyme concentrations. Sodium linoleate emulsions to which no lipoxidase had been added served as controls.

TABLE I

THE OXIDATION OF SODIUM LINOLEATE (0.015 *M*) BY SOYBEAN LIPOXIDASE

Soybean lipoxidase, mgm.	O_2 consumed (μl.) in 60 min.
0.1	103
0.5	312
1.0	472
1.5	530
2.0	600

The results (Table I) showed a relatively large uptake of oxygen by sodium linoleate in the presence of lipoxidase, although the uptake was not directly proportional to enzyme concentration. The lack of linear relationship must be taken into consideration when enzyme activity is estimated. In the absence of enzyme there was no significant oxidation of the solutions. This enzyme was therefore an active oxidative catalyst for linoleate.

TABLE II

THE EFFECT OF HEMIN ON THE OXIDATION OF SODIUM LINOLEATE (0.032 *M*)

Oxidation period, min.	O_2 uptake (μl.)			
	Concentration of hemin (moles per liter)			
	1.5×10^{-5}	3.8×10^{-5}	7.7×10^{-5}	1.5×10^{-4}
10	130	186	220	7
20	211	328	432	10
30	279	450	615	13
50	362	594	847	89
60	403	655	960	383

The Catalytic Effect of Hemin on Linoleate Oxidation

The relation between the extent of oxidation of the sodium linoleate and the amount of hemin used as catalyst was investigated. The methods already described were used and oxygen uptake was measured at 10 min. intervals over a total period of 60 min. The results (Table II) show a gradual increase in oxygen uptake with time for amounts of hemin up to $7.7 \times 10^{-5} M$. However, with 1.5×10^{-4} *M* there was a marked inhibition of oxygen uptake for a lag period of about 50 min., after which oxidation took place rapidly. The amount of hemin used subsequently was such as to avoid this induction period.

TABLE III

EFFECT OF SODIUM LINOLEATE CONCENTRATION ON THE INDUCTION PERIOD FOR HEMIN CATALYSIS (1.53×10^{-4} MOLES HEMIN PER LITER)

Oxidation period, min.	O_2 uptake (μl.) Concentration of sodium linoleate		
	0.016 *M*	0.032 *M*	0.048 *M*
10	−4	7	18
20	−2	10	195
30	−4	11	435
40	−2	18	671
50	1	79	921
60	1	354	1253
75	1	785	1508

Effect of Concentration of Sodium Linoleate on the Induction Period with Hemin

Table III shows the effect of sodium linoleate concentration on the induction period observed with hemin catalysis. Using 1.53×10^{-4} *M* hemin as catalyst, there was a very pronounced induction period in the oxidation of 0.016 *M* sodium linoleate and after one and one quarter hours there was no significant catalytic activity. In the presence of 0.032 *M* sodium linoleate there was an induction period of 40–50 min., followed by rapid oxygen uptake. In the presence of 0.048 *M* sodium linoleate there was no observable induction period.

Inhibition of Hemin Catalysis

Several compounds which are known to form complexes with the hemin molecule were tested for their inhibitory effects on oxidative catalysis. Compounds used were sodium fluoride (0.2 *M*), sodium thiocyanate (0.3 *M*), sodium azide (0.2 *M*), and potassium cyanide (0.2 *M*). Sodium linoleate (0.032 *M*) was used as substrate, hemin (1.5×10^{-4} *M*) was added, the pH was adjusted to 9.0 with borate buffer, and oxygen uptake was measured in 10 min. intervals up to two hours.

Potassium cyanide (0.2 *M*) completely inhibited hemin catalysis of linoleate oxidation in 60 min., in longer oxidation periods the cyanide inhibitory

effect decreased; the other substances had no effect. The experiment was repeated with the same compounds at pH 7.0 instead of 9.0. At the lower pH, fluoride, thiocyanate, and azide again had no inhibitory effect but at 60 min. the inhibition by cyanide was reduced to 67%.

Cyanide Inhibition of Catalysis by Other Heme-pigments

The heme-containing pigments, hemoglobin, myoglobin, metmyoglobin, cytochrome-*c*, and catalase are known to catalyze linoleate oxidation and are present in animal tissue in relatively large amounts (7, 13, 18). The effectiveness of potassium cyanide as an inhibitor for the catalytic action of these substances was investigated. The quantities of heme-containing substances tested exceed the amounts which would be present in tissue extracts used in the assay. The results (Table IV) showed that the catalytic action of all of these substances was completely inhibited by appropriate amounts of

TABLE IV

CYANIDE INHIBITION OF LINOLEATE* OXIDATION CATALYZED BY HEME CONTAINING SUBSTANCES

Catalyst, mgm.	Concentration of KCN (moles per liter)	% inhibition			
		Time (min.)			
		20	30	40	60
Hemoglobin					
1.5	0.004		100		92
2.5	0.007		98		98
3.0	0.007		100		100
Myoglobin					
5.0	0.20	100		100	
5.0	0.33	100		100	
Metmyoglobin					
5.0	0.20	96		92	
5.0	0.23	100		100	
Catalase					
1.0	0.0053		100		100
5.0	0.007		100		100
Cytochrome-c					
0.1	0.20		100		94
0.3	0.40		100		100

* *Sodium linoleate, 0.015 M.*

potassium cyanide. Hemoglobin and catalase were more sensitive to potassium cyanide than either myoglobin or cytochrome-*c*. Cytochrome-*c* was the most difficult of these pigments to inhibit. One tenth of a milligram of this substance required approximately 0.20 *M* potassium cyanide to completely inhibit its catalytic action. This amount of potassium cyanide completely inhibited 3.0 mgm. of hemoglobin, and 5.0 mgm. of metmyoglobin, myoglobin, and catalase respectively.

Another experiment showed that the sum of the individual catalytic effects of each of the heme-containing pigments was approximately two thirds of their collective effect. However the amount of potassium cyanide required to inhibit their collective catalytic action was the same as the sum of the amounts required to inhibit each individually. Approximately 0.4 *M* potassium cyanide inhibited all of these catalysts when present in amounts normally expected under assay conditions (22, 10, 16).

Effect of Potassium Cyanide on Soybean Lipoxidase

For purposes of assay it was necessary to determine whether lipoxidase activity could be detected in the presence of amounts of potassium cyanide sufficient to inhibit heme catalysis.

Various amounts of potassium cyanide were added to a solution of sodium linoleate (0.015 *M*) containing 0.1 mgm. lipoxidase (0.24 units by Bergström and Holman assay (5)) and the percentage inhibition was measured after a 60 min. reaction period. Concentrations of 0.066, 0.198, and 0.330 molar potassium cyanide had no effect on lipoxidase activity. Concentrations of 0.462 *M* and 0.600 molar cyanide caused 7% and 18% inhibition respectively. It is possible to detect increasingly smaller amounts of enzyme in the presence of decreasing amounts of potassium cyanide. However, the amount of potassium cyanide required to inhibit all heme pigments in 3–5 gm. of animal tissue was estimated to be about 0.4 *M*. In the presence of 0.4 *M* potassium cyanide, 0.1 mgm. of the lipoxidase was not inhibited and could be detected. Hence in an average tissue sample of 4 gm. approximately 0.06 units of lipoxidase per gm. could be detected.

To show that it was possible to detect activity in the animal tissue extracts, several experiments were carried out in which soybean lipoxidase in amounts as low as 0.06 per gm. units was added to cyanide-inhibited extracts of various tissues. Since all of the added enzyme was detected in all tissues tested, the assay system was considered suitable for lipoxidase detection in animal tissues.

Application of Assay Method to Detection of Animal Lipoxidase

Six different rabbit tissues were assayed for lipoxidase activity. Duplicate extracts of each tissue from each of two rabbits were incubated with 0.33 *M* potassium cyanide for 20 min. and then mixed with sodium linoleate (0.15 *M*). Oxygen uptake was measured over a 60 min. period and compared with control samples without cyanide.

The inhibition of catalytic activity in cyanide treated samples (liver, 96%; kidney, 99%; brain, 96%; spleen, 97%; lung, 93%; and heart 89%) strongly suggested that there was no lipoxidase in these tissues, with the exception of lung and heart. When, however, lung and heart tissues were assayed in the presence of 0.4 *M* cyanide, catalytic activity was inhibited 97% and 94% respectively. Although the inhibition value obtained with heart tissue might suggest a slight lipoxidase activity, the incomplete inhibition of activity in the other tissues was attributed to an estimated 5% error inherent in the method.

Some animal tissues which are of importance in the meat industry were also investigated. The materials used were three different samples of bacon adipose tissue, cured unsmoked bacon, and uncured bacon and two samples of beef adipose tissue. One milliliter of each extract (equivalent to 5 gm. of original tissue) was completely inhibited by 0.198 *M* cyanide. This amount of cyanide was much less than that required to inhibit measurable amounts of lipoxidase. Tissue residues which remained after preparation of the extracts were examined and found to be free of lipoxidase activity.

Discussion

The experimental results presented here showed that lipoxidase action at pH 9.0 can be differentiated from heme pigment catalysis of sodium linoleate oxidation by using cyanide to inhibit the activity of heme-containing substances. Cyanide eliminated the catalytic action of hemoglobin, myoglobin, cytochrome-*c*, and catalase in a borate medium at pH 9.0. These results are in agreement with those reported by Khan (9) and by Barron *et al.* (4). The inability of Watts and Peng (22) to obtain cyanide inhibition of the peroxidation of lard by hemoglobin, may be explained by the fact that their measurements were made after a period of 3–14 days and in a medium of pH 7.0. In the present study, the most effective inhibition of linoleate oxidation was obtained within the first hour of the reaction and at pH 9.0.

Application of the assay method to animal tissues strongly suggested that no lipoxidase was present. The oxidative catalysts in the extracts were effectively inhibited by cyanide and appeared to be heme compounds. These results agree with the recent findings of Tappel (17, 19, 21) and Watts and Peng (22) who could not demonstrate lipoxidase in animal tissue. Khan's reported separation of a lipoxidase from herring muscle (9) must be considered dubious since the enzyme preparation was active only in the presence of a heme-containing activator. From cured bacon adipose tissue, Reiser (12) isolated a hemoglobin-free extract which catalyzed oxidation of fats. However, neither Tappel (18) nor the present authors have been able to prepare hemoglobin-free extracts by Reiser's method. Hence Reiser's conclusion that the active oxidizing agent was a lipoxidase-like enzyme is questionable.

Certain reservations must be made in applying data obtained on soybean lipoxidase to animal lipoxidase; if the latter exists, it may differ from the enzyme of plant origin. Consideration must also be given to suitable methods

of extracting the enzyme from the animal tissues. However, in the present study, assays of several of the extracted residues failed to show any lipoxidase activity. While the absence of lipoxidase from the various animal tissues was not conclusively proved, the weight of evidence indicated that lipoxidase, if present, was in such small amounts as to play an insignificant part in unsaturated fatty acid oxidation.

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CARBOHYDRATES IN THYROGLOBULIN AND THE LENS CAPSULE[1]

BY L. UJEJSKI[2] AND R. E. GLEGG

Abstract

The carbohydrate content of thyroglobulin and the lens capsule was studied. Paper chromatography of hydrolyzates was used for qualitative investigation and the carbazole – sulphuric acid method for quantitative estimations of carbohydrates. Thyroglobulin contains 4.2% carbohydrate made up of galactose, mannose, and fucose, while the lens capsule contains 11.8% carbohydrate made up of galactose, glucose, mannose, and fucose.

The present investigation was undertaken to study the carbohydrate components of two important biological entities, namely, thyroglobulin and the capsule of the lens of the eye. Previous work on thyroglobulin indicated the presence of 2.2% hexosamine (2) and 3.45% carbohydrate of unknown nature (11). On the other hand, the lens capsule was thoroughly investigated by Pirie (10) who showed the presence of about one per cent hexosamine and 9–10% carbohydrate (orcinol method) identified as galactose and glucose by paper chromatographic analysis of hydrolyzates. However, methods of hydrolysis and chromatography recently developed in this laboratory (5, 6) revealed the presence of other monosaccharides than those previously described in the lens capsule. The present report describes these results as well as those obtained for thyroglobulin. These materials were also analyzed quantitatively for hexosamine and non-hexosamine carbohydrate.

Experimental and Results

Thyroglobulin was prepared from beef thyroids by the method of Derrien, Michel, and Roche (3). The capsule of the lens was removed from beef eyes, cleaned of all adhering material by wiping the inner and outer surfaces thoroughly, stirred in cold water overnight to remove soluble materials, and dried.

In order to identify the monosaccharide units, 100 mgm. thyroglobulin and 50 mgm. of lens capsules were hydrolyzed in the presence of a cation exchange resin (Permutit Q) for 48 hr. (5). The hydrolyzates were evaporated to dryness and the residues dissolved in 0.1 ml. water. Six microliters of the solutions were used for chromatography (5, 6). The chromatogram (Fig. 1) showed spots corresponding to galactose, glucose, mannose, and fucose in the lens capsule, and galactose, mannose, and fucose in thyroglobulin.

Hexosamine was determined by the method of Elson and Morgan as modified by Blix (1). Quantitative sugar estimations were carried out with the carbazole–sulphuric acid technique (4) modified by Seibert and Atno (12) and Holzman *et al.* (8).

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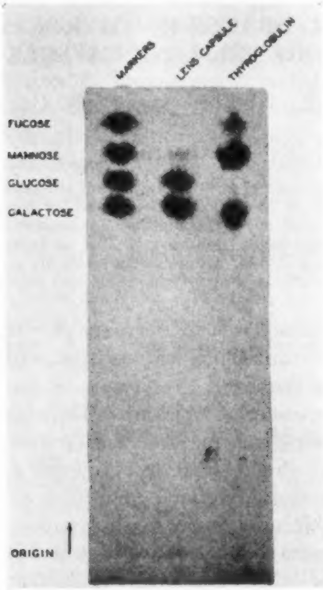

FIG. 1. Paper chromatogram of the hydrolyzates of thyroglobulin and the lens capsule.

Galactose, glucose, mannose, and fucose are present in the lens capsule. The spot for fucose is not seen clearly on the photograph, but could be easily seen on the original chromatogram. Galactose, mannose, and fucose are present in thyroglobulin.

The amount of sugar present in the lens capsule and thyroglobulin was obtained from standard carbazole – sulphuric acid curves for glucose : galactose (1 : 1) and galactose : mannose (1 : 1), respectively. The concentrations of hexosamine and non-hexosamine (carbazole) sugar are given in Table I.

TABLE I

% HEXOSAMINE AND NON-HEXOSAMINE SUGAR IN THYROGLOBULIN AND THE LENS CAPSULE

	Hexosamine	Sugar (Carbazole)
Thyroglobulin	2.3	4.2
Lens capsule	1.4	11.8

Discussion

The concentrations of hexosamine and non-hexosamine sugar are in fair agreement with those found by previous workers for thyroglobulin (2, 11) and the lens capsule (10). However, the present chromatographic study has revealed the presence of mannose and fucose in the lens capsule in addition

to glucose and galactose previously identified by Pirie in hydrochloric acid hydrolyzates (10). The identification of mannose and fucose in the present investigation is probably due to the use of a different type of hydrolysis medium employing a cation exchange resin. Galactose, mannose, and fucose appear to be the aldose units of the carbohydrate moiety of thyroglobulin.

The monosaccharide constituents of the lens capsule are similar to those recently found in a wide variety of connective tissues and derivatives (7). The large amount of carbohydrate found in these materials (Table I) is considered to be responsible for the intense periodic acid – Schiff reactions observed in histological sections of the thyroid colloid and the lens capsule (9).

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CALCIUM ABSORPTION AND VITAMIN D[1]

By B. B. Migicovsky and J. W. S. Jamieson

Abstract

The rate at which Ca^{45} administered as an oral dose appears in blood and bone of chicks is markedly affected by vitamin D. Absorption of Ca^{45} from an intramuscular dose is not influenced by either vitamin D or the calcium content of the diet. The interaction of the amount of Ca^{45} in the oral dose and vitamin D on the amount of calcium absorbed indicated that the vitamin D effect on the absorption is a function of the quantity of calcium presented for absorption. Absorption experiments carried out with chicks which were fed diets of varying calcium content showed that vitamin D enabled the chicks to adapt the capacity to absorb calcium to different dietary intakes of calcium.

Introduction

Knowledge of the mode of action of vitamin D has been reviewed by Nicolaysen and Egg-Larsen (4). Although the "primary" effects of the vitamin have been established, neither the initial effect nor its mode of action has been defined satisfactorily. It is generally agreed that the initial site of action is on either the calcium absorption mechanism or the bone. The effect at both sites has been demonstrated repeatedly. The nature of the vitamin effect, increased absorption, and mineral deposition is such that a change in one can elicit a change in the other.

It is hoped that detailed studies of the absorption and deposition mechanisms may aid in establishing the initial target for vitamin D action.

Hansard *et al.* (1) found that the ability of rats to absorb calcium was increased after a short period of time on a low calcium diet. Nicolaysen (3) observed that rats were able to adapt the ability to absorb calcium, and concluded that this adaptation ability was associated with vitamin D. This led to the proposal (4) of an "endogenous factor", which is responsible for regulating the efficiency of absorption and is inoperable in the absence of vitamin D.

In this report we are presenting some studies on calcium absorption with chicks, using the Ca^{45} method of measuring relative absorption as described by Migicovsky and Nielson (2).

Experimental

One-day-old chicks were taken at random and fed an A.O.A.C. rachitogenic diet either with or without vitamin D. When vitamin D was given, it was mixed into the diet at the rate of 1 unit per gram feed. The calcium absorption measurements were made on two- or three-week-old chicks.

The first experiment was a study of the rate at which an oral dose of Ca^{45} appeared in blood and bone and the effect of vitamin D thereon. At two weeks of age one half of the groups were given vitamin D in the diet. When

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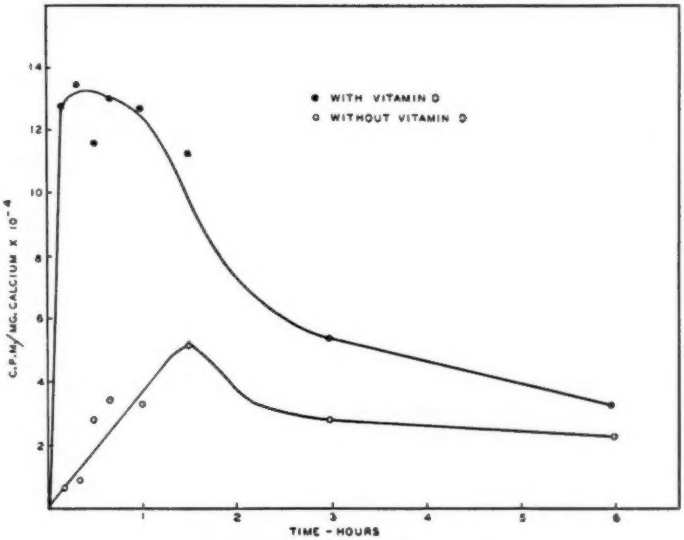

FIG. 1. Specific activity of blood calcium vs. time.

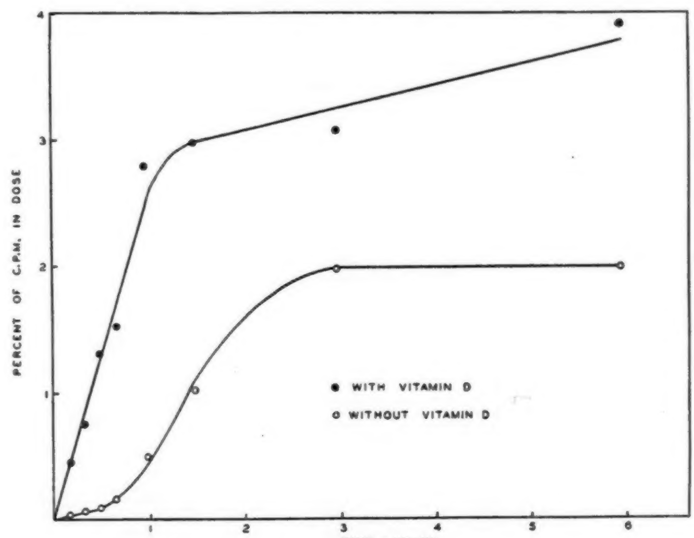

FIG. 2. Accumulation of an oral dose of Ca^{45} in tibia vs. time.

the chicks were three weeks of age, feed was removed. After 24-hr. starvation, all the chicks were given an oral dose of Ca^{45} equivalent to 5.0 mgm. calcium and 4.2×10^6 counts per minute. At varying intervals after the dose of Ca^{45}, groups were bled and the left tibiae removed. The analytical data for blood and bone are shown in Figs. 1 and 2, respectively.

The data illustrate that vitamin D exerts a positive effect on the rate at which calcium appears in the blood, that is, the rate of calcium absorption is increased by the presence of vitamin D.

The amount of Ca^{45} appearing in bone is a reflection of the calcium concentration in the blood in the intervening time. Previous work by Migicovsky and Nielson (2) showed that vitamin D did not affect the appearance of Ca^{45} in bone from an intramuscular dose, and a straight line relationship existed between Ca^{45} in the intramuscular dose and the Ca^{45} in bone. Data in Table I substantiate this finding and, in addition, illustrate that the calcium content of the chicks' diet prior to the 24-hr. starvation period does not affect the Ca^{45} content of bone after an intramuscular dose. A summary of the analysis of variance of the data in Table I is shown in Table II.

TABLE I

EFFECT OF DIETARY CALCIUM AND VITAMIN D ON PERCENTAGE OF INTRAMUSCULAR DOSE OF Ca^{45} IN TIBIA*

Diet		Vitamin D	No vitamin D
Ca, %	P, %	% Ca^{45} dose in tibia	
0.30	0.99	4.69	4.95
0.53	0.97	4.59	4.60
1.25	0.95	4.54	4.48
2.18	0.90	4.27	4.38

* *Each value represents the mean of three groups of three chicks per group.*

TABLE II

ANALYSIS OF VARIANCE OF DATA IN TABLE I

Source	Degrees of freedom	Mean square
Total	23	
Vitamin D	1	0.03[a]
Diet	3	0.25[a]
Diet × vit. D	3	0.03[a]
Error	16	0.11

[a] *Not significant at* $P = .05$.

As a result of these observations, the percentage of an oral dose of Ca^{45} appearing in bone may be taken as an index of the capacity of the organism to absorb calcium. A relative measure of the amount of calcium absorbed from any given dose is then the

$$\frac{\text{counts per minute/tibia}}{\text{counts per minute/dose}} \times 100 \times (\text{mgm. Ca/dose}).$$

The next series of experiments represented a study of the change in the absorption capacity with varying doses of calcium. Having established that vitamin D acts on the absorption mechanism by altering the rate of the calcium absorption reaction, the question arises whether the vitamin effect is a function of the amount of calcium presented to the absorption mechanism.

One-day-old chicks were taken at random and raised as described above, one half of the chicks receiving vitamin D for two weeks prior to the experiment. When the chicks were three weeks of age, the feed was removed. Twenty-four hours later the dose of Ca^{45} was administered. Twenty-four hours later the chicks were killed and tibiae were removed for analysis.

Data obtained with different doses of calcium are shown in Table III. They indicate that the percentage of Ca^{45} dose per tibia fell with increasing doses of calcium but the effect of vitamin D was comparatively constant. The relative quantity of calcium absorbed, which is percentage of Ca^{45} dose per tibia × mgm. Ca per dose, is greater with increasing doses and the effect of vitamin D thereon also increases with increasing calcium dose. Analysis of variance of the data in Table III is shown in Table IV.

TABLE III

INTERACTION OF VITAMIN D AND AMOUNT OF CALCIUM IN DOSE ON THE PERCENTAGE OF THE Ca^{45} DOSE PER TIBIA

Trial No.*	Ca/dose, mgm.	Vit. D	No vit. D	Vit. D effect†	
		% Ca^{45} per tibia		$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100$	$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100 \times (\text{mgm. Ca/dose})$
65	0.7	5.26	4.19	1.07	.0075
	1.3	4.95	4.06	0.89	.0116
	2.5	4.98	3.51	1.47	.0368
	5.1	3.80	2.96	0.84	.0428
70	0.7	5.34	3.99	1.35	.0095
	2.8	6.41	3.79	2.62	.0734
	3.6	6.46	3.58	2.88	.1037
	8.4	5.20	2.85	2.35	.1974
	11.0	5.62	2.81	2.81	.3091
67	1.3	6.39	4.96	1.43	.0186
	3.1	5.87	3.94	1.93	.0598
	7.4	4.40	2.64	1.76	.1302
	15.3	3.69	2.61	1.08	.1652
	20.0	3.14	1.77	1.37	.2740

† *Value for vitamin D treated groups minus value for vitamin D deficient groups.*
* *Each value represents the mean of four groups of three chicks per group.*

TABLE IV

ANALYSIS OF VARIANCE OF DATA IN TABLE III

Source	Expt. 65		Expt. 70		Expt. 67	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Total	31		39		39	
Vitamin D	1	9.04[b]	1	57.6[b]	1	22.9[b]
Ca^{45} dose	3	2.78[b]	4	1.8[b]	4	13.9[b]
Ca^{45} dose × vit. D	3	0.16[a]	4	0.78[a]	4	0.22[a]
Error	24	0.21	30	0.39	30	0.20

[a] *Not significant.*
[b] *Significant at P = .01.*

These experiments indicate that irrespective of the amount of calcium in the dose, vitamin D increases the proportion that is absorbed by a constant factor. This leads to the conclusion that the amount of calcium that vitamin D causes to be absorbed is a function of the amount of calcium presented to the absorption mechanism.

The final series of experiments dealt with the adaptation mechanism referred to by Nicolaysen *et al.* (5). Chicks were reared on diets varying in calcium with and without vitamin D. After a period of one week the measurement of the percentage of Ca^{45} dose per tibia was made as described above. The results are shown in Table V. Accompanying the data are the values for the amount of calcium per tibia, which is a criterion of the degree of mineralization. Analysis of variance of the data is shown in Table VI.

It is clear that the effect of vitamin D on the absorption mechanism was greater after a low calcium diet than after a high calcium diet. In the

TABLE V

INTERACTION OF DIETARY CALCIUM AND VITAMIN D ON THE PERCENTAGE OF ORAL Ca^{45} DOSE IN TIBIA*

Diet		Vitamin D		No vitamin D	
Ca, %	P, %	$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100$	Ca/tibia, mgm.	$\frac{\text{c.p.m./tibia}}{\text{c.p.m./dose}} \times 100$	Ca/tibia, mgm.
0.30	0.99	4.25	19.2	1.93	12.9
0.53	0.97	4.32	25.3	1.66	15.1
1.25	0.95	2.89	27.1	1.65	19.1
2.18	0.90	2.32	26.2	1.59	21.1

* *Each value in the table represents the mean of three groups of three chicks per group.*

TABLE VI

ANALYSIS OF VARIANCE OF DATA IN TABLE V

Source	Per cent of Ca^{45} dose/tibia		Ca/tibia	
	Degrees of freedom	Mean square	Degrees of freedom	Mean square
Total	23		23	
Vitamin D	1	18.1[b]	1	325.0[b]
Diets with vit. D	3	2.95[b]	3	38.1[b]
Diets with no vit. D	3	0.07[a]	3	41.0[b]
Error	16	0.06	16	1.78

[a] *Not significant.*
[b] *Significant at P = .01.*

absence of vitamin D, the chicks did not adapt the absorption mechanism to a low calcium intake; whereas in the presence of vitamin D this adaptation was made. It may be noted also that, irrespective of the diet, vitamin D always exerted a significant effect on the absorption mechanism.

Discussion

It is generally felt that the action of vitamin D on both the absorption and deposition mechanisms is a primary effect. Nicolaysen *et al.* (5) invoke an endogenous factor to explain the absorption effect. There is no direct evidence for the presence of an endogenous factor and we feel that a plausible explanation for the action of vitamin D can be given without recourse to such a factor.

If we may be permitted to speculate, the action of vitamin D may be explained by a reaction of the vitamin or a derivative thereof with calcium at the absorption barrier. The reaction may be pictured as Ca + vit. D→[Ca D].

With this type of a reaction in mind, the amount of calcium bound to vitamin D in the presence of an excess of the vitamin would be a function of the amount of calcium presented to the absorption mechanism. This is borne out by the results of the first series of experiments presented above.

The adaptation effect, i.e., the differential effect of vitamin D after a period of variable calcium feeding, may be explained by the degree of accumulation of the bound calcium. That is, after a week on a high calcium diet plus vitamin D, the amount of bound calcium in the blood would be higher than after a period on a low calcium diet. The increased concentration of bound calcium would serve to decrease the rate of the reaction pictured above. This could account for results obtained with experiments which illustrate the adaptation phenomenon.

The mineralization effect of vitamin D, quantitative and qualitative, could be explained by the assumption that bound calcium is deposited more

readily and in a more regular fashion. In the absence of vitamin D, where a high calcium diet yields a more mineralized but histologically abnormal bone, the deposition of calcium probably depends upon the irregular excess of calcium that is delivered to the blood via the intestinal barrier.

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AN *IN VITRO* ASSAY OF CORTICOTROPHIN[1]

By K. W. McKerns and E. Nordstrand

Abstract

The ability of corticotrophin to increase the corticoid output of rat adrenals in an isolated gland system has been developed as a useful assay method for the measurement of corticotrophin potency. The extra corticoids produced by stimulation are measured in terms of cortisone. Log dose response curves are presented for corticotrophin levels from 0.002 to 0.135 I.U./100 mgm. adrenals. A four point assay design, the precision of corticoid measurements, and the characteristics of the log dose response curves for a number of types of corticotrophin are given. With four measurements of each dose level the average lambda s/b for 20 assays was 0.209 $\pm$ 0.085 (S.D.).

Introduction

A direct action of corticotrophin stimulation in isolated adrenal systems has been demonstrated by increased corticoid output. Saffran *et al.* (11) have shown this effect on rat adrenals and have proposed an assay method (10). An *in vitro* effect of corticotrophin has also been demonstrated by Haynes *et al.* (6) on cattle adrenal cortex slices, Vogt (14) on perfused dog adrenals, and Pincus *et al.* (7) on perfused cow adrenals.

The difficulties in using this corticoid stimulating effect of corticotrophin in an isolated gland system have been due to the high corticoid output of the unstimulated isolated glands and the very variable output of different glands when stimulated with corticotrophin.

We have found in preliminary experiments that the corticoid production of an isolated unstimulated adrenal gland system appears to vary with the general metabolic condition of the rats. In addition, the corticoid output obtained by corticotrophin stimulation is related to the endogenous or unstimulated activity and is high for a given dose of corticotrophin when the endogenous level is high. As incubation proceeds, the corticoid output from corticotrophin stimulation gradually drops off as substrate in the adrenals becomes depleted. The rate of depletion is faster the higher the corticotrophin level. If, however, the rats are kept free of stress due to extremes of temperature and humidity, and in a quiet room for several days, their halved adrenal glands will usually respond satisfactorily to a dose of corticotrophin in the range of 0.005 to 0.135 I.U./100 mgm. adrenal tissue.

A preincubation of the adrenals for 30 min. in buffer without added corticotrophin is necessary to remove preformed corticoids. We have also found that a prestimulation with a small amount of standard corticotrophin reduces some of the variability between different glands. Increasing the preincubation time without corticotrophin to one or one and one-half hours has the same effect.

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Contribution from the Research and Development Laboratories, Canada Packers Ltd., Toronto, Ont.

This report describes a four point assay design that controls the variables so that a good estimate of corticotrophin potency in terms of a standard can be made in one working day.

Materials and Method

The standard corticotrophin was U.S.P. Provisional Reference Standard. The materials assayed included crude corticotrophin, purified corticotrophin prepared by mild acid extraction, complexing with metallic ions, and solvent partitioning; crude glacial acetic acid extracted corticotrophin, and oxycel purified corticotrophin.

Female Sprague–Dawley rats (90–125 gm.) are used. The bisected adrenal glands are incubated with shaking at 37° C. in buffer medium. Krebs Ringer bicarbonate pH 7.4 buffer medium, Umbreit *et al.* (13) equilibrated with 95% oxygen and 5% carbon dioxide is favored though the phosphate buffer is also satisfactory. Calcium is critical, (Birmingham *et al.* (2)) and is kept at 0.375 mM. calcium by using 1.83% calcium chloride instead of 1.22% calcium chloride.

The methanol or methylene chloride used as solvent for ultraviolet absorption measurements is reagent grade, triple distilled, and checked for absorption against water at 225, 240, 255, and 260 mμ in the model DU Beckman spectrophotometer with a photomultiplier attachment.

The chloroform used to extract the corticoids from the buffer medium is double distilled, the lower and higher boiling point material is discarded.

The filter paper used to remove the last traces of moisture from the solvent is extracted before use with chloroform in a Soxhlet.

Corticoid Estimation

Saffran *et al.* (11) used the calculation of Allen (1) to estimate the corticoid output of an isolated adrenal gland system in terms of the absorption at 240 mμ caused by the α, β unsaturated ketonic group. There was a significant correlation between the spectrophotometric corticoid estimations and those obtained by the Spiers and Meyer eosinophile reduction method (12).

Elliott *et al.* (5) have made a comparison between the ultraviolet absorption estimation of total corticoids and the estimation of α ketol adrenal cortical steroids by the reduction of 2, 5-diphenyl-3-(4-styryl-phenyl)-tetrazolium chloride. Chromatographic separations were also made of extracts from adrenal incubates and measured by both methods.

Following the suggestion of Birmingham *et al.* (3) we have used the formula of Allan (1):

$$a.\quad CD_1 = OD_{240} - \frac{(OD_{225} + OD_{255})}{2}$$

in conjunction with a second formula:

$$b.\quad CD_2 = OD_{240} - OD_{260}$$

as the best estimation of adrenal steroids measured in the presence of interfering substances having an absorption curve concave to the wavelength axis.

The corticoid output of the adrenal glands is expressed as gamma cortisone acetate. A standard curve was made for a solution of cortisone acetate in methanol, the gamma steroids being plotted against calculated optical density. The slope of curve *a.* = 16.666 (reciprocal = 0.060) and that of curve *b.* = 30.25 (reciprocal = 0.033).

The unknown corticoid values are obtained as follows:

c. corticoids/100 mgm. adrenals

$$= \frac{(CD_1 \times 0.060) + (CD_2 \times 0.033)}{2} \times \frac{100}{\text{adrenal weight}} \times 4$$

The ultraviolet measurements are made in 4 ml. of methanol. However, in the tables to follow the corticoid value per milliliter is used and may be multiplied by four to obtain corticoids/100 mgm. adrenals.

Methylene chloride was also used as a solvent for extraction and measurement of corticoids (9). A standard curve was made for a solution of cortisone acetate in methylene chloride.

Precision of Extraction of Added Cortisone Acetate

Cortisone acetate was dissolved in Krebs Ringer bicarbonate buffer so that each milliliter contained 20 gamma. Two milliliter portions of this solution were extracted once with 10 ml. chloroform, the chloroform evaporated, and the corticoids measured in 4 ml. of methanol.

Two milliliter portions of the same cortisone acetate solution were extracted with 4 ml. of methylene chloride and the corticoids estimated.

The corticoid values for eight different extractions with each solvent are as follows:

For chloroform—methanol		For methylene chloride	
8.91	9.01	9.60	9.95
9.06	9.18	10.00	10.05
8.83	8.50	9.65	10.10
8.28	8.88	9.50	10.00
Mean = 8.83 ± 0.28 gamma/ml.		Mean = 9.86 ± 0.23 gamma/ml.	

The blank values obtained when the buffer itself is extracted with chloroform are 0.43 ± 0.06 gamma/ml. The blank is zero for methylene chloride.

Assay Design

For each assay 32 female rats are used—eight litters of four litter mates. This design is repeated for any one sample and weighted means taken until the desired limits of error are achieved.

A four point assay design is set up in the following manner:

The adrenals are removed from rats that have been lightly anesthetized with ether and then with sodium pentobarbital. The glands are trimmed free of fat and bisected. The four pieces of adrenal tissue thus obtained from each rat are placed into four sectors of a buffer-moistened filter paper in a covered petri dish. One rat from each of the eight litters contributes pieces in this fashion to the four sectors of the filter paper. The adrenal tissue in each sector is weighed and placed in a Warburg flask containing 2 ml. of buffer solution previously equilibrated with 95% oxygen, 5% carbon dioxide. There is the equivalent of adrenal tissue from two rats in each flask. The adrenal tissue distributed in the four flasks constitutes the "series" of the assay design. This distribution is repeated so that there are four series containing litter mate tissue. The 16 flasks of the design can be handled in one day.

The flasks are shaken in a Warburg apparatus or equivalent for 30 min. at 37° C. under 95% oxygen and 5% carbon dioxide. The buffer solutions are sucked off and discarded. The gland tissue in each flask is then stimulated by 0.01 I.U. U.S.P. Provisional Reference Standard Corticotrophin per 100 mgm. tissue in 1 ml. buffer solution for one hour. These buffers are also discarded and the glands are washed with 2 ml. of fresh buffer per flask. The adrenal tissue is now conditioned for the actual stimulation with the corticotrophin under study. Two milliliters of buffer containing corticotrophin at the desired dose level are added to each of the four flasks in series one and repeated for the other three series. The flasks are incubated for one hour and the buffers drawn off into 20 ml. tubes made by sealing the bottoms of 20/40 ground glass joints. Another one hour incubation is carried out with the same dose levels of corticotrophin in each flask and the buffers saved again. Duplicate determinations can thus be made from each flask and the mean values used.

The buffers are extracted once with 10 ml. of chloroform by shaking the glass stoppered tubes 100 times. The tubes are then centrifuged, the water layer carefully removed, and the chloroform filtered through Whatman No. 1 paper into 40 ml. tubes having outside ground 14/40 joints. Two or three milliliters of chloroform are used to wash each centrifuge tube and filter paper.

The chloroform is evaporated to dryness at 50° C. using a water pump. Each tube is fitted to a manifold having ground glass joints to receive the tubes. A further one hour evaporation at 50° C. to remove the last trace of chloroform is done using a mechanical pump.

The residue is finally dissolved in 4 ml. of methanol and the corticoid value per ml. calculated from ultraviolet absorption measurements. When methylene chloride is used, the buffer solutions are extracted once with 4 ml. After separation of the aqueous phase the methylene chloride extract is used directly for ultraviolet absorption measurements.

Results

Dose Response Curves

The dose response relationship for U.S.P. Provisional Reference Standard is shown in Table I.

TABLE I

RELATION BETWEEN DOSE AND CORTICOID OUTPUT. THE CORTICOID OUTPUT IS EXPRESSED AS GAMMA CORTICOIDS/ML.

Series	Unstimulated	Doses ACTH/100 mgm. adrenals		
		0.002 I.U.	0.008 I.U.	0.016 I.U.
1 (a)	6.4	8.1	10.4	10.4
(b)	5.1	8.4	9.1	10.3
2 (a)	4.9	6.4	7.8	8.5
(b)	4.0	6.5	7.1	9.6
3 (a)	5.2	9.1	9.1	9.7
(b)	4.6	9.7	10.3	10.1
4 (a)	6.1	7.5	8.7	8.4
(b)	5.3	8.0	9.0	9.7
Totals	41.6	63.7	71.5	76.7
Means	5.2	7.8	8.9	9.6
Increase over unstimulated level		50%	71%	85%

$s = 0.50$; $\bar{b} = 1.77$; $\lambda = s/b = 0.28$.

The statistical methods of analysis used are described by Burn, Finney, and Goodwin (4).

Table II shows the dose response relationship for the Reference Standard repeated in another system of 32 rats for a different dose range.

TABLE II

RELATION BETWEEN DOSE AND CORTICOID OUTPUT. THE CORTICOID OUTPUT IS EXPRESSED AS GAMMA PER ML. AND AS THE MEAN OF TWO STIMULATIONS

Series	Doses ACTH/100 mgm. adrenals				Total
	0.005 I.U.	0.015 I.U.	0.045 I.U.	0.135 I.U.	
1	7.08	8.40	9.73	13.17	38.38
2	9.40	9.17	11.43	15.18	45.18
3	6.95	9.46	10.54	11.82	38.77
4	6.42	8.56	9.75	10.47	35.20
Total	29.85	35.59	41.45	50.64	
Mean	7.46	8.90	10.36	12.66	

$s = 0.88$; $\bar{b} = 3.63$; $s/b = 0.242$.

Assay Examples

The corticoid response for a partly purified corticotrophin sample run against the U.S.P. Provisional Reference Standard is given in Table III. The unknown was run at an assumed potency of 2.5 I.U./mgm.

TABLE III

ASSAY No. L 57

The response figures are given as gamma corticoids/ml.

Series	Doses: Standard, 0.005 I.U./100 mgm.	Doses: Standard, 0.015 I.U./100 mgm.	Doses: Unknown, 0.005 I.U./100 mgm.	Doses: Unknown, 0.015 I.U./100 mgm.	Total
1	5.80	6.74	5.10	6.45	24.09
2	7.37	8.38	7.03	8.88	31.66
3	8.13	10.46	8.39	8.93	35.91
4	8.67	9.09	8.42	9.51	35.69
Total	29.97	34.67	28.94	33.77	
Mean	7.49	8.67	7.24	8.44	

TABLE IV

ANALYSIS OF VARIANCE FOR THE DATA OF TABLE III

Nature of variation	Degrees freedom	Sum of squares	Mean square
Total	15	30.66	s^2
Between doses	3	5.92	
Between series	3	22.88	
Error	9	1.86	0.2067

The ratio of mean squares (F) as a test for parallelism and validity for assay L57 = 0.051 and is well below the significance level of 5.1 for probability of 0.05. The mean slope $\bar{b} = 2.50$ ($b_s = 2.46$, $b_u = 2.53$), $\log R = \bar{1}.9035$ and the potency = 2.00 I.U./mgm. $\lambda = s/b = 0.185$.

The approximation of the error of the log ratio of the potencies ($S \log_R$) is calculated according to the example given by Pugsley (6).

$$95\% \text{ limits on } \log_R = \log R \pm 2.26\ (S \log_R)$$

(for 9 degrees of freedom $t = 2.26$) $= \bar{1}.9035 \pm (2.26 \times 0.091)$

$R = 0.8007$

95% limits on $R = 1.286$ to 0.4986

Further examples of assay data obtained for several types of corticotrophin are given in Table V. The examples are a consecutive series of 20 assays and a true expression of the average result to be expected. All of them are preliminary assays run at an assumed potency level based on the method of preparation and the expected potency.

TABLE V

ANALYSIS OF FOUR POINT ASSAYS ON A NUMBER OF DIFFERENT CORTICOTROPHIN SAMPLES. FOUR DETERMINATIONS WERE MADE ON EACH DOSE LEVEL

Test No.	λs	$\bar{\lambda}$	s	b_s	b_u	$\bar{b}$	F ratio	Potencies assumed	I.U./mgm. determined
41	.251	.263	.447	1.90	1.48	1.70	0.20	2.0	0.69
42	.226	.233	.482	2.13	2.00	2.07	0.016	2.0	2.43
43	.254	.218	.480	1.88	2.50	2.20	0.066	2.0	2.60
44	.250	.244	.805	3.21	3.38	3.30	0.010	4.0	4.78
45	.270	.282	.599	2.22	2.02	2.12	0.026	4.0	1.55
46	.230	.278	.551	2.40	1.55	1.98	0.053	50.0	53.6
47	.187	.207	.335	1.79	1.45	1.62	0.235	50.0	83.0
48	.222	.227	.590	2.66	2.53	2.60	0.010	2.5	1.14
49	.095	.089	.260	2.94	2.55	2.75	0.519	2.5	1.55
50	.350	.425	.496	1.42	0.91	1.17	0.238	2.5	0.37
51	.258	.219	.275	1.07	1.44	1.25	0.405	3.0	1.32
52	.453	.333	.576	1.27	2.19	1.73	4.38	2.2	1.01
53	.140	.191	.475	3.38	1.59	2.49	3.26	2.0	1.26
54	.210	.133	.281	1.34	2.87	2.11	6.84	2.2	1.88
55	.047	.066	.167	3.52	1.53	2.54	32.46	3.0	1.70
56	.221	.222	.813	3.68	3.62	3.65	0.011	1.7	1.55
57	.185	.182	.454	2.46	2.53	2.50	0.051	2.5	2.00
58	.189	.236	.745	3.94	2.36	3.16	1.03	2.2	1.20
59	.087	.109	.373	4.27	2.53	3.41	4.99	2.2	1.45
60	.200	.165	.690	3.46	4.87	4.18	0.95	2.5	2.25
61	.215	.159	.601	2.80	4.74	3.78	2.37	2.5	2.43

λs = lambda of the standard.
$\bar{\lambda}$ = mean lambda of standard and unknown.
s = standard deviation.
b_s, b_u, $\bar{b}$ = slope standard, unknown, and mean respectively.
F ratio = variance ratio of slope difference. The significance level for 9 degrees freedom for probability of 0.05 is 5.1. All assays should have an F value below this level of significance of slope difference.

All samples, with the exception of numbers 46 and 47, were assayed against the U.S.P. Provisional Reference Standard at 0.005 I.U./100 mgm. adrenal tissue for the lower dose and 0.015 I.U. for the higher dose. Samples 46 and 47 were oxycel purified and assayed against a glacial acetic acid extracted house standard. Test number 56 was done on a glacial acetic extracted unknown run against the U.S.P. Provisional Reference Standard. All other unknown preparations were dilute mineral acid extracted preparations.

In tests 56 to 61 inclusive, methylene chloride was used as the solvent for extraction and measurement of corticoids, replacing chloroform and methanol.

Discussion

The assay has been set up on the basis of using a four point series of pooled adrenal tissue and four series litter mate related. The standard error (s) is much lower within the series than between the series and this is allowed for in the statistical analysis.

Considering the complexity of the assay system, the average s/b ratio of 0.209 ± 0.085 (S.D.) on only four measurements per dose is good, and compares favorably with other assay systems for corticotrophin. Preliminary work indicates a close correlation with the intravenous Sayer's ascorbic acid depletion method. However since there are several physical forms of corticotrophin in clinical use, complete correlation with other assay methods will require many more comparisons.

The assay technique would be more useful and economical if the "series" could be extended so that more dose levels were available for a standard and an unknown, or so that several corticotrophin types could be compared in one assay system. We are currently developing an improved design.

There would appear to be an advantage in a method that relates corticotrophin activity to its ability to stimulate extra corticoid output since it is the corticoids that are responsible for the clinical effect.

Since lambda varies greatly with the steepness of the response curve, it is very important that the animals be free of continuous stress that will deplete the adrenal reserves. In hot humid summer weather this usually means that air conditioned quarters are necessary to maintain good adrenal reserves.

Acknowledgments

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THE CONCENTRATION OF ELECTROLYTES IN NORMAL HUMAN SALIVA[1]

By J. A. Hildes and Marion H. Ferguson

Abstract

Parotid saliva was collected into graduated tubes by fitting small cups over the parotid papillae. The sodium, chloride, and bicarbonate concentrations and the pH of parotid saliva increased with increasing rates of secretion, but the potassium and phosphate concentrations remained constant at secretion rates greater than 0.5 ml. per minute. Although the latter two ions were always at concentrations higher than serum levels, the parotid juice was always hypotonic but decreasingly so as the rate of flow increased. There was appreciable variation between individuals and also in the same subject from time to time. No difference was noted between the two gustatory stimuli used, dilute acetic acid and glucose candy. The potassium concentration in parotid saliva was higher than in mixed submaxillary and sublingual saliva. Continuous recording of the rate of secretion on a smoked drum showed cyclic variations from minute to minute, indicating that timed collections over a period of minutes give mean rather than actual rates of flow.

Introduction

It has been known for many years that the sodium, chloride, and bicarbonate concentrations in the saliva of dogs and rabbits increase when the secretory nerves to the glands are stimulated (7, 15) and that the potassium concentration is not so affected (6, 4, 9). In a recent paper Thaysen, Thorn, and Schwartz (12) have reported similar results in three human subjects in whom secretion was stimulated by the subcutaneous injection of Mecholyl.

Other factors may also affect the electrolyte concentration of saliva. Babkin (1) concluded, mainly on the evidence obtained by Baxter (2), that different oral stimuli produce secretions of differing composition even though the mean rates of secretion remain constant.

In the experiments reported here the parotid glands of normal human subjects were reflexly activated by gustatory stimuli to observe the effect of rate of secretion on the electrolyte composition of saliva, to define the range of variation to be found in normal subjects, and to ascertain the effect of different stimuli. As many reports in the literature deal with mixed saliva (11, 16), some comparisons were also made between parotid juice and the mixed saliva from the submaxillary and sublingual glands.

Methods

The Subjects used were medical students, all but Subject A being male. They attended the laboratory, usually in the afternoon, having had no special preparation.

Experimental Procedures

Small double-walled brass cups were fixed over both parotid papillae and held in place by negative pressure applied to the outer rims (10). The saliva

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Contribution from Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Manitoba.

was drained from the inner chambers through plastic tubes into graduated receptacles for measured time intervals. "Resting" and stimulated secretions were collected and analyzed for sodium, potassium, chloride, and phosphate content. In a few experiments the parotid juice was collected under oil for the determination of bicarbonate content and pH.

The juice was collected separately from the two sides to compare the simultaneous secretions from right and left glands.

To compare the results of different stimuli, dilute acetic acid and glucose candy were used. The latter stimulus was held on the midline of tongue. The acetic acid stimulus was delivered to the midline of tongue by a fine plastic tube, connected with a syringe, to give a constant stimulus during each collection period. Various concentrations from 0.1% to 1.0% were used to achieve a satisfactory range of secretory rates.

Serial tests were run on the same subjects at daily or weekly intervals to establish the variation in individuals from day to day and the variation between individuals.

In a few experiments the collecting vessels were connected with air-tambour systems to give a continuous recording of the secretion rates on a smoked drum.

In two subjects the parotid juice was collected from both glands as above and the mixed saliva from the remaining salivary glands was collected over a timed period by spitting into a beaker. The volume was then measured and the saliva analyzed for sodium and potassium.

Analytical Methods

Sodium and potassium were estimated in a Barclay flame photometer with a barrier layer type photo cell against an internal standard (3); chloride by the method of VanSlyke and Hiller (13); phosphate by the method of Fiske and Subbarow (5); pH in a Model G Beckman pH meter; and carbon dioxide in the VanSlyke–Neill manometric apparatus (14).

Results

General Pattern

Fig. 1 shows the results of experiments on Subjects A, E, and F stimulated with graded concentrations of dilute acetic acid. This method of stimulation produced a satisfactory range of secretory rates from 0.02 to 2.7 ml. per minute. The concentrations of sodium, chloride, and bicarbonate were low in the resting secretion but those of potassium and phosphate were greatly in excess of serum levels. The potassium concentration fell as secretory rates of 0.5 ml. per minute were approached but thereafter remained constant at a level four to six times that of the serum. The phosphate concentration showed a similar relationship to secretory rate with a constant level three times that of the serum. The sodium and chloride concentrations increased with increasing rates of secretion but did not reach normal serum levels. However, the bicarbonate concentration, at rates above 1.0 ml. per min., exceeded the serum concentration. The pH increased from 6.0 to 7.8 as the secretion rate increased from 0.5 to 2.1 ml. per min.

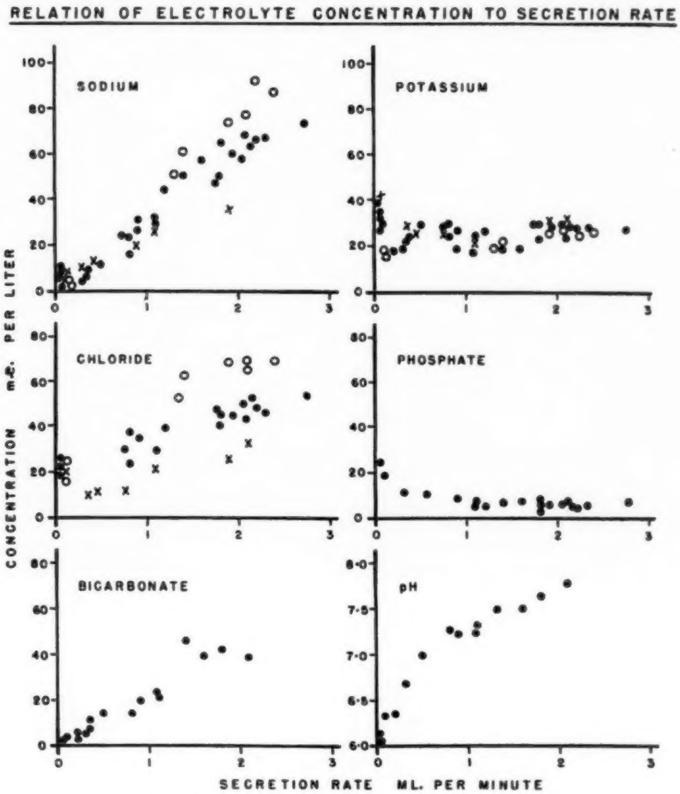

FIG. 1. The concentrations of sodium, chloride, bicarbonate, potassium, and phosphate and the pH of parotid juice plotted against the rate of secretion in three subjects stimulated with graded strengths of dilute acetic acid. The juice was collected under oil for estimations of bicarbonate and pH.

Cyclic Variations of Secretory Rate

Fig. 2 shows the simultaneous rate of secretion from right and left glands recorded on a smoked drum over a three-minute period of constant stimulation. The slopes of the lines at any time reflect the two secretory rates at that time. It is apparent, particularly in the lower tracing, that the glands were not secreting at a constant rate but in irregular cycles of 10 to 30 sec. with variable periods of decreased activity. The changes in rate were not synchronous on the two sides and, therefore, it is not likely that they were due to small changes in the strength of stimulus although this possibility cannot be entirely excluded. Similar fluctuations were found to a varying degree in most experiments where this method of recording was used.

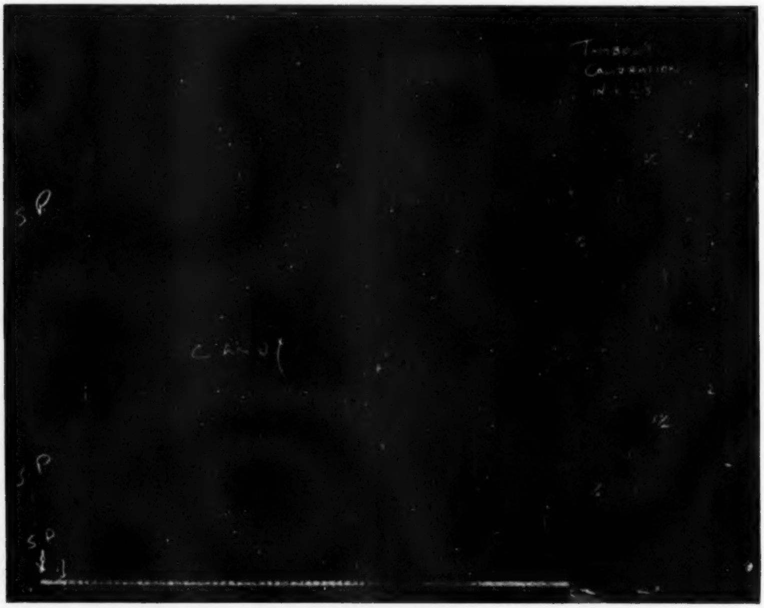

FIG. 2. Graphic record of secretion rate from right and left parotid recorded on a smoked drum through air–tambour systems. Recording of secretion rate was started at points marked S.P. by closing an open side arm, and ended with the return of the writing arm to the base line, which was accomplished by opening the side arm to atmospheric pressure. The tambours were calibrated for volume in 0.5 ml. steps at the right of the tracing. The time marks are at 10 sec. intervals. Glucose candy was used as the stimulus. The irregular spurts of secretion are seen in both tracings, particularly the upper one.

TABLE I

COMPARISON OF BILATERAL SIMULTANEOUS PAROTID SECRETION RATES

Subject	No. of tests	Mean difference as % of higher rate	Rt > Lt	Rt = Lt	Rt < Lt
Acetic acid stimulus					
A	13	27.5 ± S.D. 8.7	100	0	0
B	30	9.4 ± S.D. 7.9	23	10	67
C	12	14.9 ± S.D. 8.7	8	0	92
D	60	14.5 ± S.D. 7.5	10	3	87
26 others	32	22.3 ± S.D. 18.5	47	3	50
Candy stimulus					
D	16	11.6 ± S.D. 14.0	44	19	37
26 others	28	20.8 ± S.D. 16.0	57	11	32

Note: The simultaneous rates of secretion from right and left parotid glands measured in ml. per minute are compared. The difference is expressed as a percentage of the higher rate of secretion. The last three columns indicate the percentage of the trials in which right or left glands had a faster rate or were equal.

Comparison of Right and Left Glands

Table I shows the frequency and extent of variation between right and left parotid secretory rates measured simultaneously. It is interesting to note that in Subject D the left gland usually secreted at a higher rate than the right when stimulated with acetic acid but this did not hold when the candy stimulus was used. Despite the wide differences in simultaneous secretory rates, the relationship between rate of secretion and sodium concentration was the same for the right and left glands. This is shown in Fig. 3 for Subject D stimulated with the acetic acid stimulus. The same held for Subjects A, C, and I who were also tested in this way.

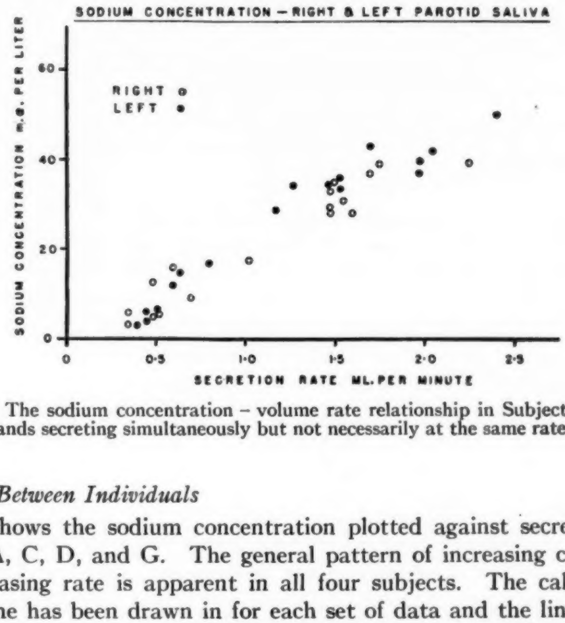

FIG. 3. The sodium concentration – volume rate relationship in Subject D for right and left glands secreting simultaneously but not necessarily at the same rate. (Table I).

Variation Between Individuals

Fig. 4 shows the sodium concentration plotted against secretion rate in Subjects A, C, D, and G. The general pattern of increasing concentration with increasing rate is apparent in all four subjects. The calculated best straight line has been drawn in for each set of data and the lines compared statistically by the method of the Standard Error of the Difference Between Regressions (8). With the exception of two comparisons, G vs. C which had a P value of $< .02$, and C vs. A which showed no significant difference, the lines differed one from the other with a P value of $< .01$.

Day to Day Variations in One Individual

In Subjects A, C, D, and I the relationship between sodium concentration and secretory rate was compared in tests run on different days. The calculated regression lines for Subject D on three days are shown in Fig. 5. In this subject and also in Subjects C and I the regression lines are somewhat divergent but not significantly so. In Subject A, who was tested four times, two of the lines were significantly different from the other two.

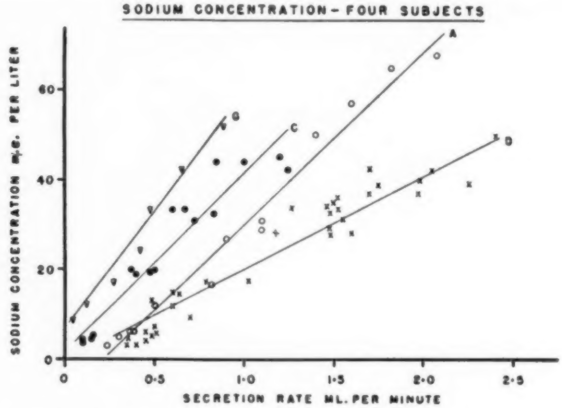

FIG. 4. The variation in the sodium concentration – volume rate relationship in four subjects—A, C, D, and G. The calculated regression lines are statistically different except that for Subject C vs. Subject A.

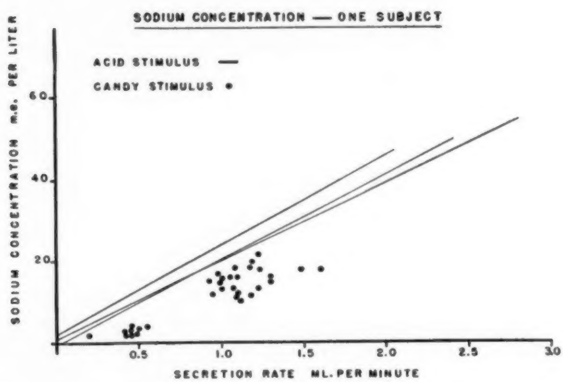

FIG. 5. The calculated regression lines for three test runs at weekly intervals on Subject D with acetic acid stimulus are shown. The closed circles represent the sodium concentrations found with the glucose candy stimulus in the same subject a week after the last acetic acid trial.

The variation between individuals and the day to day variation in the same individual are shown in Table II with respect to potassium concentration. As the potassium concentration is independent of rate except at low rates of secretion (Fig. 1), the results shown in Table II are those with secretory rates greater than 0.5 ml. per min.

The resulting means range between 21.7 and 28.4 meq. per liter except one which was 18.1. Not only is there appreciable variation between individuals but also a small, but often statistically significant, variation from day to day in the same individual.

TABLE II

VARIATION IN CONCENTRATION OF POTASSIUM IN PAROTID JUICE (MEQ. PER LITER)

Subject	Trial			
	1	2	3	4
A	27.9 ± .55* (6)	28.4 ± .47 (6)	23.8 ± .27 (4)	18.1 ± 3.5 (4)
B	27.7 (4)	27.4 ± .13 (12)	28.1 ± .03 (14)	
C	22.6 ± .28 (5)	24.9 ± .12 (9)	22.2 ± .01 (6)	
D	22.4 ± .06 (18)	21.7 ± .03 (10)	23.8 ± .15 (29)	21.7 ± .02 (22)

Note: Secretion rate in all tests greater than 0.5 ml. per min. Number of samples shown in parentheses.
* *Standard error of mean.*

The Effect of Different Stimuli

Glucose candy was used as a stimulus to compare the secretion that candy produced with that obtained with the acetic acid stimulus. The sodium results obtained with candy stimulation in Subject D are shown in Fig. 5 as closed circles along with the calculated regression lines for three test runs on the same subject with the acid stimulus. Because of the grouping of the secretory rates with the candy stimulus it did not seem justified to calculate a line for these points. However, it can be seen that with this stimulus, as with the acid stimulus, there is an increase in sodium concentration with increasing rates of secretion. The points for the candy stimulus all fall below the mean lines for the acid stimulus. However, there is a considerable overlap of the candy and acetic acid results. The latter are not shown in Fig. 5. With the candy stimulus the mean potassium concentration with rates of secretion over 0.5 ml. per min. was 21.8 ± S.E. 0.03 meq. per liter. This result falls within the range of variation for potassium concentration in the same subject stimulated by the acid stimulus (Table II).

Comparison of Parotid and Mixed Submaxillary and Sublingual Saliva

In experiments on Subjects H and I the sodium and potassium concentrations of parotid and mixed submaxillary and sublingual saliva were compared. Secretion rates ranging from 0.1 to 3.2 ml. per min. were achieved. The sodium concentration in the mixed submaxillary and sublingual saliva showed the same general trend of an increase in concentration with increasing rates of secretion. It is difficult to compare the concentration in this pooled saliva from four glands with that in the juice obtained from single parotid glands as there is no assurance that each gland was contributing equally to the volume of secretion.

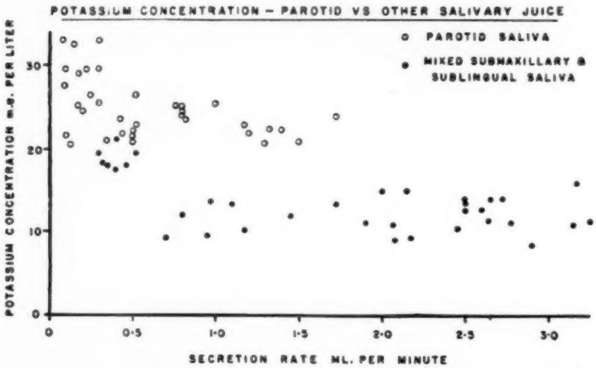

FIG. 6. A comparison of potassium concentrations in simultaneously collected saliva from the parotid glands and from the submaxillary and sublingual glands.

The potassium concentration of the mixed submaxillary and sublingual saliva also seemed to behave in a similar fashion to the potassium concentration in the parotid juice, in that there was no dependence of potassium concentration on rate of secretion at secretory rates higher than 0.5 ml. per min. Fig. 6 shows the potassium concentration from the parotid saliva compared to that of mixed submaxillary and sublingual saliva in Subject H. It can be seen that the concentration of potassium in the mixed saliva is distinctly lower than the potassium concentration in parotid saliva. Similar results were found in the other subjects tested.

Discussion

By the use of Lashley cups (10) the parotid gland secretion can be collected and measured with ease in the normal intact human subject without contamination from other body fluids. For this reason the parotid seems the ideal gland in which to study the effects of various conditions on glandular secretion. The results described here may indicate the range of variation that can be expected in normal subjects, and the necessity for considering the source of the saliva and its rate of secretion.

The use of mean secrĕtion rates rather than actual rates is a possible source of error. It is obvious that if, during the period of collection, the secretion rate varies widely, the mean rate will not have the same relationship to the sodium concentration as when the rate of secretion is constant throughout the collection period. The variation in volume of secretion illustrated in Fig. 2 is not only uncontrolled but is not determined in most experiments. This may, in part at least, explain the variations in the results obtained between individuals and in the same individual from day to day. The effects of such fluctuations in rate may also have some bearing on the interpretation of the results of Baxter (2) who used pooled five minute samples in his investigation of the effect of different stimuli on the electrolyte composition of saliva. Our

results do not indicate any real difference between dilute acetic acid and candy stimulation on the composition of parotid juice.

The general pattern of the results is in agreement with the findings in animals (6) in that potassium concentration is independent and sodium concentration is directly dependent on the rate of salivary flow. This statement does not hold for potassium concentration at secretion rates below 0.5 ml. per minute. This also has been noted by other observers and attributed by Babkin (1) to water reabsorption from the ducts. Our results with respect to the sodium concentration – volume rate relationship differ from those reported by Thaysen *et al.* (12), also in human subjects, in that these authors consider there is a decreasing change in sodium concentration with increasing secretory rates. Although the secretion rates reported here are not quite as high as theirs, our data seems to fit a straight line relationship within the range of secretory rates achieved. The variation between individuals may have some bearing on this apparent discrepancy as the results of Thaysen *et al.* (12) are shown only as the pooled data from four experiments on three subjects.

Although the parotid juice is always hypotonic, within the range of secretory rates studied, the degree of hypotonicity decreases as the rate of secretion increases. In terms of osmotic work per unit volume of saliva the energy required at high secretion rates is lower than it is at low secretion rates.

Acknowledgments

We are grateful to the students who were the subjects for these experiments and particularly to Dr. W. E. Shepherd, Mrs. M. Gard, Messrs. D. R. Parliament, G. E. Boyd, and P. J. Portnuff who helped us with various aspects of the project. This work was in part supported by the National Research Council of Canada and by the Manitoba Institute for the Advancement of Medical Education and Research.

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THE EFFECT OF HEPARIN ON THE ENDOGENOUS LIPEMIA PRODUCED BY PROTRACTED CORTISONE TREATMENT IN THE RABBIT[1]

By P. Constantinides, G. Szasz, and M. Darrach

Abstract

The clearing effect of heparin on lipemias of alimentary or renal origin is well established. This study was undertaken in order to find out whether heparin will also clear a lipemia of hormonal origin. Lipemia was produced in rabbits by prolonged cortisone treatment. Heparin failed to clear the cortisone-induced lipemia and it did not influence the concomitant hypercholesterolemia, although it exhibited an anticoagulant effect. Despite the long standing lipemia, no atherosclerosis developed in the cortisone treated animals. Some of the possible interpretations of these findings are discussed.

Introduction

It is well known that heparin clears the lipemic turbidity produced by fat alimentation. Hahn's (12) original findings in dogs have since been confirmed by numerous investigators in several species, including man. Heparin has also been reported to clear the lipemia of nephrosis in humans (2, 13) and in rats (22).

The lipemia-clearing effect of heparin is accompanied by a rapid degradation of "giant" lipoprotein molecules (with high S_f rate) to smaller molecules (with lower S_f rate) (10), by a drop in chylomicron count and size (27, 1), and by increased fat hydrolysis (3, 21). Since the lipemic turbidity of the serum is proportional to its content of large lipoprotein molecules (19), it is generally believed that the heparin-induced clearing of lipemia reflects the disappearance of these macromolecular units from the blood stream.

All these effects of heparin are indirect, i.e. they are mediated by a so-called "lipemia-clearing factor" which is an enzyme system activated by heparin (1, 24, 17).

Heparin and certain heparinoids have also been found to diminish *in vivo* the hypercholesterolemia (5, 14, 8) and to inhibit the atherosclerosis (10, 5, 14, 11) produced by cholesterol feeding in rabbits.

Since protracted cortisone treatment causes pronounced lipemic turbidity and hypercholesterolemia (16), it was considered of interest to find out whether heparin affects such a hormonal lipemia in the same way as it affects one of alimentary or renal origin.

[1] *Manuscript received November 5, 1954.*

Contribution from the Anatomy and Biochemistry Departments, University of British Columbia, Vancouver, B.C. The results of this study were presented at the 7th Annual Meeting of the Western Section, Medical Division, National Research Council, in Saskatoon on February 5, 1953, and abstracted in the Proceedings of the XIX International Physiological Congress in Montreal, August 1953.

Materials and Methods

Twenty-two New Zealand White rabbits of mixed sex and of an average initial body weight of 2.5 kgm. were injected with cortisone three times weekly for 64 days. At the end of this period, the 14 animals that developed persistent lipemia were selected for further experimentation while the others were discarded.

The 14 rabbits with persistent lipemia were then divided into two groups of seven animals each (four females and three males), and care was taken to distribute the lipemia values equally among the two lots. In both groups the cortisone treatment was continued for another 31 days, but the animals of one group were henceforth given heparin, in addition to cortisone.

A third group of eight normal rabbits provided untreated controls.

Thirty-one days after commencement of the heparin treatment (or 95 days after commencement of the cortisone treatment) the experiment was terminated with the sacrifice of all animals.

The serum turbidity of the cortisone and the cortisone plus heparin groups was measured thrice before and thrice after commencement of the heparin treatment. The serum turbidity of the untreated controls was estimated only terminally. All blood samples were obtained from the ear veins and centrifuged. The sera were diluted 1 : 3 with distilled water and their turbidity was recorded as logarithmic scale "A" reading (function of optical density) in a Fisher Model AC photoelectric colorimeter, against a water blank, using a red filter. In the heparin treated group, blood was always sampled two hours after the last heparin injection.

Serum total cholesterol was estimated terminally with the Sperry (23) method in all animals.

Cortisone acetate (Merck) was injected intramuscularly (in the hind legs) at a dosage of 6 mgm./kgm., three times weekly.

Heparin sodium (Connaught) was injected subcutaneously at a dosage of 10 mgm. per injection, twice daily.

The formalin fixed left adrenal, left kidney, liver, and spleen were weighed and examined histologically. In addition, the aortae were examined macroscopically for evidence of atherosclerosis.

All animals were maintained on standard chow and tapwater ad libitum.

Results

A. Lipemic Turbidity

As can be seen in Table I, cortisone caused the appearance of a marked lipemic turbidity, which was not affected by heparin. There was no significant difference between the mean turbidity values of the cortisone and cortisone plus heparin treated groups after commencement of heparin treatment. A turbidity increase of borderline significance ($P = .02$) occurred in the heparin treated group after 18 days of heparin injections.

It should be noted that the anticoagulant effect of heparin was present in all instances. The average terminal clotting time in the cortisone treated

TABLE I

MEAN LIPEMIC TURBIDITY VALUES ± THEIR STANDARD ERROR

The turbidity figures are colorimetric scale readings and, therefore, functions of the serum optical density. The days are numbered with reference to the commencement of cortisone injections. Heparin injections were started on the 65th day

No. of animals	Group	Before heparin			After heparin		
		26th day	49th day	64th day	69th day	83rd day	95th day
8	I. Untreated controls						1.2 ± 0.03
7	II. Cortisone	6.3 ± 2.8	9.5 ± 3.0	12.1 ± 1.9	8.3 ± 3.4	8.3 ± 3.2	8.1 ± 3.3
7	III. Cortisone + heparin	7.3 ± 1.6	8.4 ± 3.6	14.3 ± 3.0	9.7 ± 1.7	23.3 ± 5.7	10.8 ± 3.9

group was 7.6 ± 1.06 min., whereas the terminal clotting time in every animal of the cortisone plus heparin treated group was more than one hour.

The failure of heparin to clear the cortisone-induced lipemia was also demonstrated in a short-term experiment. On the 65th day of cortisone treatment, the effect of a single intramuscular injection of 20 mgm. heparin was recorded in two cortisone treated animals. In these two animals the serum turbidities just prior to heparin injection were 8.1 and 16.2 colorimetric scale units respectively. Two hours after the injection, the turbidity values were 8.2 and 16.1 respectively. In both cases, the postheparin blood remained fluid for more than six hours.

B. Blood Cholesterol

Heparin had no effect on the cortisone-induced hypercholesterolemia. The terminal serum cholesterol values (expressed as milligrams cholesterol per 100 ml. serum) were as follows:

Control, 62.5 ± 10.5; cortisone, 814.3 ± 244; cortisone plus heparin, 725.0 ± 293.

C. Organ Weights

As can be seen in Table II, heparin did not interfere with the cortisone-induced liver and kidney enlargement or spleen and adrenal atrophy.

TABLE II

MEAN ORGAN WEIGHTS ± THEIR STANDARD ERROR

	Liver (gm./kgm. terminal body wt.)	Kidney (gm./kgm. terminal body wt.)	Spleen (mgm./kgm. terminal body wt.)	Adrenal (mgm./kgm. terminal body wt.)
Control	35.3 ± 2.4	2.81 ± 0.19	519 ± 64.3	36.0 ± 1.42
Cortisone	72.6 ± 4.2	6.01 ± 0.48	314 ± 51.5	21.4 ± 2.74
Cortisone + heparin	81.0 ± 1.6	5.05 ± 0.41	278 ± 32.0	26.6 ± 4.22

D. Histological Examination of the Liver and the Kidney

Variable degrees of disseminated small round cell infiltrations and scarring were observed in the kidneys of most cortisone treated animals. Heparin did not seem to influence these lesions.

The livers of both the cortisone and the cortisone plus heparin treated group showed histological signs of massive glycogen storage.

E. Aortae

In view of the long-standing lipemia in the cortisone treated groups, the aorta of every rabbit was examined macroscopically from the valves to the iliac bifurcation. No evidence of atherosclerosis was found in the aortae of the lipemic animals. Small "spontaneous" plaques occurred with equal frequency in the ascending aortae of all three groups.

This was supported by the absence of coronary lesions from routine microscopic sections of the heart of all animals.

Discussion

Amounts of heparin or heparinoids similar to those used in this study consistently clear the lipemia and decrease the hypercholesterolemia resulting from cholesterol–oil feeding in the same strain of rabbits (5, 11). Since heparin displayed an anticoagulant without a lipemia-clearing effect in the present experiment, it is possible that the specific lipemia produced by cortisone is not susceptible to the clearing action of heparin, or that cortisone inhibits the formation of "lipemia-clearing factor" in response to heparin.

Since we have no data on the lipoproteins or the "lipemia-clearing factor" in this experiment, both hypotheses are tentative and will have to be tested experimentally.

The general changes of serum lipids after cortisone injections and after cholesterol feeding appear to be similar. Both treatments have been reported to increase the serum lipoproteins, fatty acids, cholesterol, and phospholipids (20, 19, 6, 9).

Pierce and Bloom (19), however, found that cortisone increases mainly the lipoproteins of the S_f 80–400 class, affects the S_f 40–80 class only slightly, and has no effect on the S_f 20 and lower class. They therefore proposed that cortisone imposes a metabolic block at the S_f 40–80 class of lipoproteins, leading to an accumulation of lipoproteins of higher S_f rate. Their contention was supported by the fact that cessation of cortisone treatment was followed by a serial conversion of the accumulated S_f 80–400 lipoproteins into materials of successively lower S_f rate, until the normally occurring S_f 3–12 class was reached.

It has been postulated (15, 19) that the circulating lipoproteins of high S_f rate are continuously converted into lipoproteins of lower S_f rate, in the course of a physiological transport process which is greatly accelerated by heparin (10). The suggested blocking of this serial lipoprotein degradation by cortisone might, therefore, account for the failure of heparin to clear the lipemia produced under such conditions.

The second possibility may be supported by the observation that prolonged cortisone treatment of the rabbit causes glycogen infiltration of the pancreatic islets and blood sugar changes resembling those of alloxan diabetes (16). Since diabetes inhibits the production of lipemia-clearing factor by heparin (7), it is possible that cortisone prevented the clearing of lipemia through its diabetogenic action. Further support for this assumption might be found in our report that cortisone pretreatment of rats decreases the formation of lipemia-clearing factor after heparin injection (4). Direct proof of lipemia-clearing factor inhibition in cortisone-treated rabbits will be required, however, before such an explanation can be considered.

While the mechanism of the observed facts remains obscure, it is of interest to note that a number of other "antilipemic" agents fail to affect lipemias of endogenous origin. Thus, dextran sulphate has been reported to clear the lipemia of oil-fed rats but not that of pregnant animals (3). Iodides will suppress a hypercholesterolemia resulting from cholesterol feeding but not

one produced by thyroidectomy (18). Finally, thyroxine prevents an exogenous lipemia much more than one caused by stilbestrol treatment (18).

The absence of atherosclerotic lesions despite the long standing lipemia in the cortisone-treated animals is in line with the findings of Cook *et al.* (6) and it has been expanded by subsequent reports of an inhibitory effect of cortisone on cholesterol atherosclerosis (9, 25, 26). Here again, only tentative explanations can be offered at the moment.

According to Jones *et al.* (15), the most atherogenic lipoproteins belong to the S_f 12–50 class, while those of lower or higher S_f rate are not atherogenic. If this is correct, it would appear that the bulk of lipoproteins accumulating in the blood during cortisone treatment belong to a non-atherogenic moiety (19).

Another interesting possibility (also considered by other authors (9, 26)) is that cortisone prevents the penetration of lipids into the aortic wall by decreasing the permeability of the intima.

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